COMMON MESSENGER MOLECULES AND CELL TYPES DEMONSTRATING NEUROENDOCRINE-IMMUNE INTERACTIONS IN THE CHICKEN

A Dissertation

by

CHERIE MORGAN OUBRE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Microbiology

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Approved as to style and content by:

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ABSTRACT

Common Messenger Molecules and Cell Types Demonstrating

Neuroendocrine-Immune Interactions in the Chicken. (May 2005)

Cherie Morgan Oubre, B.S., Lamar University

Co-Chairs of Advisory Committee: Dr. L. Berghman
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The aim of this study was to identify common messenger molecules used in both the immune and the neuroendocrine systems in birds, and to shed light on a cell type within the bursa of Fabricius that has historically been postulated as a potential neuroendocrine-immune link, the bursal secretory dendritic-like cells (BSDC). An immunocytochemical approach was used to identify neuroendocrine cell populations in the thymus, pituitary and bursa of Fabricius in the chicken. Molecular confirmation of the neuroendocrine cell marker, chromogranin A (CgA) in the thymus tissue of the chicken was reported. Previously the serine protease inhibitor, ovoinhibitor, was localized in bursal follicles, specifically the cortico-medullary border region. The presence of ovoinhibitor was identified and confirmed in the chicken pituitary by this study. Continued focus on the neuroendocrine-immune interactions in chicken immune tissue narrowed the study around the BSDC population. The BSDC are a component of the stromal, non-lymphoid cellular environment of the bursa of Fabricius and are thought to play a role in B-cell maturation and differentiation. They are located mainly along the cortico-medullary border of the bursal follicles in the same area as the majority of the

ovoinhibitor-positive cell population. During attempts to isolate the BSDC population by flow cytometry and laser capture microdissection, a cell culture method was developed that enriched the BSDC population by 10-fold. This enriched population was used to evaluate protein product secretion following lipopolysaccharide (LPS) challenge and compared to *in vivo* challenge with live *Salmonella*. For the first time, up-regulation of the pro-inflammatory cytokine IL-12 was documented in the chicken following *in vivo* challenge. In addition, the gene expression of serine protease inhibitors was markedly decreased in the adherent cell population following LPS stimulation. As a result of this research a novel method for the enrichment of an adherent population, including the BSDC, was developed, providing a valuable tool for the analysis of this population during immune stimulation.

To my wonderful Koko

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CHAPTER I

INTRODUCTION

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The chicken immune system

The chicken has played an important role in the understanding of the immune system. In the last 65 years, the chicken has become an excellent and well-regarded model for the study of the humoral immune system. The concepts of the innate and acquired immune systems originated by discoveries made in the bursa of Fabricius. The ability to study both primary lymphoid organs, the thymus and bursa of Fabricius in their cellular context has been a valuable tool for immunologists. Since the discovery of the bursa of Fabricius, it has been found that several other species contain gut-associated lymphoid tissues (GALT) as important components for B-cell development, for example, sheep rely on ileal Peyer's Patches (Reynaud et al., 1991) and rabbits utilize the appendix (Lanning et al., 2000).

Thymus

The chicken thymus, like that of other species, is the site of naïve T-cell proliferation and maturation. It is located along the neck of the chicken, containing 2

This dissertation follows the style and format of Immunity.

rows of seven lobes, one on each side of the neck (Jeurissen et al., 1994b). The lobes are further divided into lobules, which are the functional units of the thymus, separated by septa and connective tissue. Each lobule has an outer cortex and inner medullary area. In the thymus, arteries are found in the medullary area of the lobes and non-myelinated nerve fibers are located around each blood vessel (Chan, 1992; Jeurissen et al., 1994b). The outer cortical region is populated with large numbers of epithelial cells and double positive ($CD4^+$ and $CD8^+$) T-cells. The T-cells mature as they migrate from the cortex to either $CD4^+$ or $CD8^+$ cells. T-cells expressing TCR1 ($\gamma\delta$) are found in the cortico-medullary border and T-cells expressing TCR2 are located in the medullary region of the lobule (Jeurissen et al., 1994b). A small population of B-cells has been described in the medulla in germinal centers along with follicular dendritic cells that trap immune complexes and mononuclear phagocytes (Jeurissen et al., 1988; Jeurissen et al., 1994b). The blood flow is restricted to the medulla of the thymus allowing environmental antigens to enter the medullary area, only where the mature T-cells and germinal centers are located.

Stem cells located near the thoracic aorta give rise to T-cell precursors that colonize the bone marrow in embryonic development (Cooper et al., 1991). During embryogenesis the thymus is populated in waves, unlike the population of the bursa, which is populated during a small window of time (Le Douarin, 1978). The waves occur at day 15, 18, and 21 of embryogenesis (Cooper et al., 1991).

Pre T-cells are produced and circulate through the blood until they reach the thymus where chemoattractant molecules, including β_2-macroglobulin, are produced to

encourage thymic colonization (Champion et al., 1986; Le Douarin, 1978; Slimane et al., 1983). The γδT cells ($TCR1^+$) are the first T-cells expressed in the thymus, occurring around day 15 of embryogenesis during the first wave of colonization (Bucy et al., 1990). These cells lack the co-stimulatory CD28 molecule and require additional stimulation from αβT-cells in order to respond (Arstila, 1996; Arstila et al., 1993). The $CD8^-$ subset of the γδT cells make up approximately 50% of the circulating T-cells in the adult chicken (Sowder et al., 1988), while the $CD8^+$ γδT-cells are located mainly in the spleen and intestinal epithelium (Arstila and Lassila, 1993). The $CD8^+$ γδT-cells have the ability to respond to mitogen stimulation *in vitro* without the help of the αβT-cells (Arstila et al., 1995). The mature αβT-cells express on their cell surface either $CD4^+$ and function as helper T-cells or $CD8^+$ and function as cytotoxic T-cells. They recognize MHC class II- presented antigens and respond by cytokine release causing increased immune function (Chen et al., 1988). The αβT cell can be further subdivided into Vβ1 and Vβ2 thymocytes and are the predominantly expressed type in the second and third waves of colonization respectively (Chen et al., 1989; Chen et al., 1988). The Vβ1 subpopulation becomes the predominant αβT-cell population colonizing the periarteriolar sheaths of the spleen and the lamina propria of the intestinal lining, where the Vβ2 population is minor in comparison to the Vβ1 and is not found in the intestine (Bucy et al., 1988; Char et al., 1990).

Bursa of Fabricius

The bursa of Fabricius was first identified and named by Hieronymus Fabricius in the 16th century, supposedly serving as a sac for the deposit of semen (Adelmann, 1942; Davidson, 2003). While conducting further studies on the chicken, in the 1960s, Bruce Glick shared his bursectomized animals with another instructor, Timothy Chang, to conserve animals; as luck would have it, Chang wanted to demonstrate antibody production to his class, stimulating the chickens with *Salmonella typhimurium* "O" antigen. Much to their surprise, the chickens failed to produce the antibodies and after further investigation the connection between the bursa and humoral immunity was made (Davidson, 2003; Glick, 1987). The bursa is now regarded as the site of B-cell development and maturation in the chicken (Cooper et al., 1966; Glick, 1956; Warner et al., 1962).

Ontogeny and structure of the bursa of Fabricius

The bursa is a hollow sac-like organ branching off of the dorsal side of the cloaca. It begins formation at day 4 of embryogenesis (Edwards et al., 1975; Ratcliffe, 1989) and continues to function until sexual maturity when it involutes and regresses. During the embryonic stage, the bursa begins to develop its folds and the mesenchyme proliferates around the epithelial cells. During the follicular stage the epithelial bud formation of the bursa develops and colonization by pre-bursal stem cells takes place (Glick, 1995). Between day 11 and 12 of embryogenesis, the epithelial buds form lymphoepithelial tissue composed of recticular endothelial cells of endodermal origin, B-

cell precursors and dark cells from the mesenchyme which develop into macrophages or bursal secretory dendritic-like cells (Glick, 1984; Nagy et al., 2001). Pre-bursal stem cells colonize the bursa from day 7 of embryogenesis until day 14 embryogenesis, at which point the pre-bursal stem cells are no longer capable of entering the bursal tissue (Ratcliffe, 1989). Between day 12 and 14 of embryogenesis, the epithelial lining differentiates into interfollicular epithelium (IFE) and follicular associated epithelium (FAE) (Naukkarinen and Sorvari, 1984). The FAE is located directly above the follicle and begins functioning by absorbing luminal contents between day 19 and 20 of embryogenesis (Bockman and Cooper, 1973; Glick and Olah, 1993a; Olah and Glick, 1978). The IFE covers the bursal tissue in between the FAE and functions in mucus production into the bursal lumen (Nagy et al., 2001).

After hatching, the bursa has 10-15 plicae or folds and produces 8,000-12,000 follicles containing dense cortico-medullary border regions separating the cortical and medullary area (Glick and Olah, 1993a). Each of the follicles has its own blood supply and the lymph drainage occurs in the lamina propria between the follicles (Jeurissen et al., 1994b; Olah and Glick, 1992b). Lymphatic vessels are found immediately outside the follicles (Jeurissen et al., 1994b). The medulla contains lymphocytes, macrophages, BSDC and epithelial cells (Olah and Glick, 1987). A dense population of T-cells is found in germinal centers near the bursal duct, as well as a few cells in the lamina propria, and isolated T-cells can be found in the cortico-medullary border and outer cortex areas (Jeurissen et al., 1994b; Olah and Glick, 1992b). The cortex and medullary lymphocytes arise from the same precursor cells, but have different morphologies and

functions upon immune maturity (Pink et al., 1985; Ratcliffe, 1989). Cortical lymphocytes are smaller than the medullary lymphocytes and are rapidly dividing in the cortical region. The cortical lymphocytes account for 60% of the peripherial blood lymphocytes and they live between 2 to 3 days (Paramithiotis and Ratcliffe, 1994a). The cortical lymphocytes may continue cellular division, die of apoptosis, be released into the blood or move into the medullary region (Paramithiotis and Ratcliffe, 1996). The medullary lymphocytes on the other hand, are dividing at a much lower rate, they account for 35% of the peripherial blood lymphocytes and are much longer lived, between 2 and 3 weeks (Paramithiotis and Ratcliffe, 1993; Reynolds, 1987).

The bursal lumen is supplied with gut-derived antigens as well as external antigens from the environment due to a unique retrograde peristalsis that allows the uptake of the environmental antigens by the anal lips into the cloaca (Ekino et al., 1980; Schaffner et al., 1974). The environmental sample is moved upwards towards the bursa and some of the contents enter the lumen (Schaffner et al., 1974; Sorvari et al., 1975). The FAE take in the antigens by pinocytosis and deposits them into the medullary region of the bursal follicle where they associate with dendritic cells and potentially present antigen to the B-cells in the medullary region (Ekino, 1993; Glick and Olah, 1993b; Naukkarinen and Sorvari, 1984). It has been proposed that the fate of the medullary B-cell relies on recognition of its cognate antigen, once an antigen is recognized, the B-cell will undergo further cell division and be released into the blood as a long lived lymphocyte (Paramithiotis and Ratcliffe, 1996). This view proposes at least two types of B-cells are present in the peripheral blood lymphocyte population. The cortical derived

group is plentiful and diverse, but short-lived while the medullary derived population is longer lived and more specific, with the potential to recognize environmental antigens (Paramithiotis and Ratcliffe, 1996).

The idea of environmental up-take by the bursal medulla has been a focus of study for several years. Surgical or chemical bursectomy prior to three weeks of age leads to a marked decrease in the ability of the chicken to mount a humoral immune in response to environmental stimuli (Ekino et al., 1980; Ekino et al., 1985a; Glick and Olah, 1987a). Previous studies have investigated the cloacal route of inoculation to evaluate the bursal follicular response. Upon cloacal inoculation, the medulla of the bursa seems to take on a secondary immune function, by increasing cell division in the medullary region of the follicle as well as accessory cell recruitment and activation (de Azevedo and Betti, 1993; Glick, 1983).

B-cell development

B-cell development begins before B-cell precursors reach the bursa. At day 5 of embryogenesis, the pre B-cells are located in the para-aortic foci of basophillic cells (Dieterlien-Lievre, 1981). Around day 7 of embryogenesis, these pre B-cells are found in the blood islands of the yolk sac (Moore and Owen, 1967) as well as the spleen (Houssaint et al., 1991), bone marrow, (Ratcliffe et al., 1986; Weber and Foglia, 1980) and in the blood (Moore and Owen, 1967). The rearrangement of immunoglobulin (Ig) genes and production of cell surface Ig occurs outside of the bursa, in the peripheral tissues (Benatar et al., 1991; Reynaud et al., 1992). Pre-bursal B-cells are only found in

the developing embryo and are not present after hatch (Ratcliffe and Paramithiotis, 1990). Unlike mammals, immunoglobulin gene rearrangement does not generate a vast diversity of the resulting in-frame B-cells, due to limited numbers of functional recombination possibilities. Chicken B-cell diversity is gained in the bursa through somatic gene conversion, inserting upstream pseudogenes into V regions or V and D regions of light and heavy chains respectively (McCormack and Thompson, 1990a; McCormack and Thompson, 1990b; Ratcliffe, 2002; Weill and Reynaud, 1987). The chicken light chain contains 1 V region, 1 J region and 25 pseudogenes and the heavy chain has 1 V, 16 D, 1 J and 80-100 pseudogenes. The conversion process can occur multiple times, vastly increasing the diversity of the Ig repertoire (Weill and Reynaud, 1987). The process of gene conversion is a bursa-dependent event (McCormack et al., 1991).

B-cells in the bursa of Fabricius are likely to undergo apoptosis; only 5% of produced B-cells emigrate from the bursa due to clonal deletion (Scott, 2004). The developing B-cells require the bursal environment; when B-cells are removed from their cellular environment, the physical and chemical requirements for cell survival are lost. Studies have shown that within 6 hours, 80% of B-cells in cell culture die by apoptosis (Compton and Waldrip, 1998; Neiman et al., 1994). B-cell activating factor (BAFF), a member of the TNF family, is well characterized in mammals and has recently been identified in chickens as a B-cell survival factor, which plays a role in their survival and maturation (Mecklenbrauker et al., 2004). BAFF binds to three TNF receptors, transmembrane activator and calcium modulator and cyclophilin ligand interactor

(TACI), BAFF receptor (BAFF-R) and B-cell maturation antigen (BCMA,) and B-cells display all three receptors (Koskela et al., 2004; Schneider et al., 2004). The chicken B6 alloantigen has been shown to cause aggregation and promotion of apoptosis in developing B-cells (Funk et al., 1997). The bursal secretory dendritic-like cell (BSDC) is thought to play a role in the B-cell maturation by displaying immune complexes formed from maternal derived antibodies and environmental antigens on their surface (Glick, 1991; Yasuda et al., 2002). All of these factors work in unison to produce functional B-cells for rapid and specific humoral immune response.

Bursal secretory dendritic-like cells

Bursal secretory dendritic-like cells (BSDC) were first identified in 1978 by Olah and Glick, and have since been deemed an important part of the bursal microenvironment with a potential role in B-cell selection and differentiation (Glick, 1994; Glick and Olah, 1993a; Olah and Glick, 1986). It has been previously shown that antibodies against vimentin (Dako, clone 3B4) can be used to stain BSDCs via immunocytochemistry (Olah and Glick, 1992a; Olah and Glick, 1992b). The conjugated anti-vimentin monoclonal antibody (Mab) shows a staining pattern of single positive cells concentrated mainly around the cortico-medullary border of the bursal follicle during the first few weeks after hatching and later vimentin-like immunoreactivity becomes more prominent in the medulla. A Mab against the S-100 protein also stains the BSDC population, but only after antigen stimulation (Gallego et al., 1992). The putative secretory product of the BSDCs and the exact relationship between the

secretory cells and the lymphopoietic cells involved in bursal follicle formation were unknown in 1993 (Glick and Olah, 1993b) and have yet to be discovered. Quantitative electron microscopy indicates that the BSDC account for approximately 0.5% of the total bursal population (Olah and Glick, 1987). The morphology of the cells is described as having an elongated cytoplasm with two processes, one containing dense cytoplasmic granules, either located near the nucleus in immature cells or near the surface of one of the processes in mature BSDC (Olah and Glick, 1987).

The origin of the BSDC cell population is thought to come from "dark" cells of the mesenchyme of bursal plicae (Nagy et al., 2001; Olah and Glick, 1985). The dark cells migrate between day 12 and 14 of embryogenesis to the epithelium through the basement membrane, inducing epithelial bud formation and acquiring the morphological characteristics of secretory cells (Bockman and Cooper, 1973; Grossi et al., 1976; Nagy et al., 2001; Olah and Glick, 1987). The precursor "dark" cells stain with an antibody against splenic dendritic cells, and are distinct from the B-cell population. They are located mainly at the cortico-medullary border, giving credit to the proposed view that the BSDCs come from the dark cells (Glick and Olah, 1993b; Olah and Glick, 1992a). The anti-vimentin Mab labels the BSDC population at day 14 of embryogenesis (Olah and Glick, 1992a) and they are morphologically is similar to the FDC found in the spleen (Glick and Olah, 1984), caecal tonsils (Olah and Glick, 1979), avian lymph nodes (Olah and Glick, 1983), and Meckel's diverticulum (Olah et al., 1984). It has been proposed that the BSDCs are a modified dendritic cell population with the potential endocrine function of aiding B-cell conversion events in the bursal follicle. They are a

distinct population from the macrophage cells in the bursa and are not phagocytic (Olah and Glick, 1987). At hatch, the BSDCs display surface related IgG molecules (Glick, 1995). Olah and Glick propose that the IgG on the BSDC surface acts as a ligand between the BSDC and the in-frame B-cell that displays IgM receptors. The binding causes BSDC to secrete factors that initiate gene conversion and replication in the B-cell (Glick and Olah, 1993a; Glick and Olah, 1993b). (Figure 1)

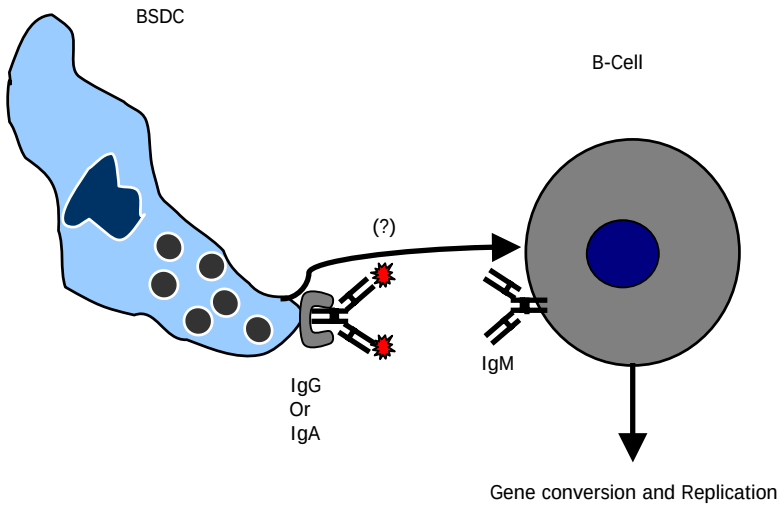

Figure 1: Proposed interactions between BSDC and B-cell. It has been hypothesized that ligand:ligand interactions between the IgG or IgA of the BSDC cell and IgM of the B-cell produce a positive signal for initiation of gene conversion and replication events (Jeurissen et al., 1994b; Olah and Glick, 1987; Ratcliffe, 1989).

Neuroendocrine – immune interactions in the bursa of Fabricius

Previously, it was believed that the neuronal, endocrine, and immune systems functioned independently of one another. We now know that each system contributes and relies on each of the other systems in order to function. Relatively little is known

currently about the neuroendocrine-immune interactions that are occurring in the bursa of Fabricius. A recent study has localized the hormone Bursin in the bursal follicle and defined it's actions to stimulate the differentiation of bursal stem cells into Bu-1 antigen-expressing B-cells (Otsubo et al., 2001). Other neuroendocrine components, the Trk family of neurotrophin receptors are composed of tyrosin kinase proteins that have also been found in lymphoid organs of mammalian species in addition to the central nervous system (Laurenzi et al., 1994; Lomen-Hoerth and Shooter, 1995). The expression of the neurotrophin receptor is directly linked to the ability to act in response to the presence of the corresponding neurotrophin (Ip and Yancopoulos, 1994; Ip and Yancopoulos, 1996). Neurotrophic factors, including neurotrophins and their receptors, are key players of the cell structure by affecting nerve growth as well as formation of neuromuscular junctions in many tissue types including the central nervous system and immune tissues (Ciriaco et al., 1997; Ip et al., 2001). The neurotrophin receptors have been recently identified by immunohistochemistry in the chicken bursa of Fabricius, indicating their presence in this immune organ and a elucidating a new area of neuroendocrine influence in the bursa (Ciriaco et al., 1997). The TrkB neurotrophin receptor has been localized in medullary secretory dendritic cells, the BSDC, in the bursa of the pigeon indicating a potential neuroendocine-immune function of these cells. TrkB neurotrophin receptors bind and are activated by brain-derived neurotrophic factor (BDNF) and neurotrophin 4/5(NT4/5), which are involved in nerve growth and survival processes (Guiton et al., 1994; Ip and Yancopoulos, 1994; Ip and Yancopoulos, 1996). Nerve growth factor (NGF) administration in ovo was reported to cause a significant increase in size of the lymphoid

follicles at day 15 of embryogenesis (Laudiero et al., 1991). NGF also prolongs bursal cell survival *in vitro* by inhibiting apoptosis (Bracci-Laudiero et al., 1993). Previous studies have identified the release of nerve growth factors by mast cells and the localization of TrkB in the ovarian follicle as well as the developing embryo (Jensen and Johnson, 2001; Skaper et al., 2001).

Recent studies have identified and localized ovoinhibitor, a protease-inhibiting protein, in the bursa of the chicken (Moore et al., 2004) as well as in the pituitary (Oubre et al., 2003) indicating a link between the immune and endocrine tissues in expression of this protein. Over 50 years ago, ovoinhibitor was originally purified from egg whites as a side product of partially purified chicken ovomucoid (Lineweaver, 1947). It is a 50 kDa multi-domain serine protease inhibitor (serpin) that was first identified and isolated by Matsushima (Matsushima, 1958). Ovoinhibitor belongs to the Kazal family of serine proteases inhibitors that specifically inhibits serine proteinases such as trypsin, chymotrypsin, subtilisin and porcine elastase (Matsuda et al., 1983; Saxena and Tayyab, 1997; Shechter et al., 1977; Vered et al., 1981; Zahnley, 1975). Immunologically, ovoinhibitor is related to the α2-proteinase inhibitor present in the serum (Barrett, 1974; Laskowski and Kato, 1980) and to vitelloinhibitor, a protease inhibitor recently purified from the yolk of hen's ovarian follicles (Sugimoto et al., 1996). The proteinase inhibitors in egg whites include ovoinhibitor as well as ovomucoid, ovostatin, cystatin and ovalbumin, and have been suggested to be involved in protecting eggs from microbial infection (Stevens, 1996) and in the differentiation of embryonic tissues, but these assumptions remain to be proven (Saxena and Tayyab, 1997). Serpins are the

major protease inhibitors found in the plasma of higher animals, where they regulate very diverse processes, including blood coagulation, fibrinolysis and inflammation (Patston and Gettins, 1994; Potempa et al., 1994; Vassalli et al., 1991) and have also been described in endocrine tissues and in the brain. Examples include kallistatin, expressed in the adrenal gland (Ecke et al., 1992) and Endopin I and II, expressed in the adrenal medulla, the pancreas and the pituitary (Hwang et al., 1994; Hwang et al., 1995; Hwang et al., 1999a). Ovoinhibitor has been demonstrated previously in the chicken oviduct and liver, where its expression is hormonally regulated by estrogens and progesterone (Stevens, 1991; Zhu et al., 2001).

Other reports about putative neuroendocrine elements in the bursa are extremely scarce. Proopiomelanocortin (POMC)-derived peptides, including β-endorphin in the bursa of young birds and ACTH and α-melanocyte-stimulating hormone (α-MSH) in birds older than 2 months were demonstrated recently by Franchini and Ottaviani using immunocytochemistry (Franchini and Ottaviani, 1999). A member of the IL-6 family, Neurotrophin-1/B-cell-stimulating factor-3 has been described as a stimulator of B-cells (Senaldi et al., 1999). Cells positive for chromogranin A, a classic neuroendocrine cell marker, can be seen after intrabursal disease virus infection of the bursal tissue (unpublished results).

Neuroendocrine components, ovoinhibitor and neurotrophin receptor, TrkB have been localized by immunohistochemisty in the BSDC population of the bursa (Ciriaco et al., 1997; Moore et al., 2004). This provides an important potential link between neuroendocrine regulation and B-cell differentiation.

Conclusion and summary

The chicken immune system is an ideal model to study the interactions of the neuroendocrine and the immune systems. The localization of the cell-mediated and humoral immune systems in the thymus and the bursa of Fabricius, respectively, makes the chicken an excellent model to investigate not only the basic function of the immune system, but also its interactions with the nervous and endocrine systems.

The bursa of Fabricius is a discrete organ that is the site of humoral immune function unique to birds, which allows the characterization of the cells involved in humoral immune maturation in their cellular context. Currently, the bursa is being studied extensively to understand the complexities of the humoral immune system development. Continued evaluation of the bursa as a model for B-cell development will also increase the understanding of interactions between the neuroendocrine and the immune system. The BSDC are likely to provide information about important signals for B-cell maturation. The presence of cells with a morphology suggestive of endocrine or neuroendocrine activity and labeled with neuroendocrine antibodies, represents yet another example of the inter-digitation of the endocrine and the immune systems.

The research described in the present manuscript is targeted toward the evaluation of the interactions between the neuroendocrine and the immune system, evaluating the localization of known neuroendocrine components, ovoinhibitor and chromogranin A, and further characterizing the putative neuroendocrine cell type, the BDSC, in immune tissues.

CHAPTER II

IMMUNOHISTOCHEMICAL ASSESSMENT OF THE NEUROSECRETORY CELLS OF THE CHICKEN THYMUS USING A NOVEL MONOCLONAL ANTIBODY AGAINST AVIAN CHROMOGRANIN A*

Introduction

Chromogranin A (CgA) is one of the best-characterized members of a family of acidic secretory glycoproteins. It is a marker of neuroendocrine cells, amine and peptide hormone and neurotransmitter containing secretory granules, also referred to as large dense-cored vesicles (Huttner et al., 1991a). Intracellularly, CgA is involved in sorting and packaging of peptides into secretory granules and in the regulation of neuropeptide and peptide hormone processing (Huttner et al., 1991b; Seidah et al., 1987; Wiedenmann and Huttner, 1989). At the same time, CgA is also a prohormone: it is the precursor protein for several bioactive peptides, including vasostatin (Zhang et al., 1999), pancreastatin (Udupi et al., 1999), and other peptide hormones with autocrine, paracrine, and endocrine activities. Overall, the CgA-derived bioactive peptides are believed to counteract the biological action of the hormones with which CgA is co-stored. This is

* Reprinted from Developmental and Comparative Immunology, Vol 28, No 4, Oubre CM, Zhang X, Clements, KE, Porter TE, Berghman LR. "Immunohistochemical assessment of the neurosecretory cells of the chicken thymus using a novel monoclonal antibody against avian Chromogranin A", Pages 337-345, Copyright (2004), with permission from Elsevier. (doi:10.1016/j.dci.2003.08.008)

especially well documented for catestatin, a potent catecholamine antagonist (Kennedy et al., 1998; Mahata et al., 2000a; Mahata et al., 2000b; Mahata et al., 1998; Mahata et al., 1997; Taupenot et al., 2000; Taylor et al., 2000).

CgA-positive cells were described for the first time as a potential link between the nervous and immunological systems by Angeletti and Hickey (Angeletti and Hickey, 1985). Additionally, CgA has received a lot of attention because of its value in the diagnosis of a wide variety of neuroendocrine tumors (O'Connor et al., 1983), including neoplasms of the thymus (Wick and Rosai, 1988). The diversity of tissues containing CgA-positive cells is enormous (including the chromaffin cells of the adrenal medulla, the parathyroid chief cells, thyroid parafollicular C cells, the pancreatic islet cells and gut neuroendocrine cells; (O'Connor et al., 1983) and is a reflection of the fact that these CgA-producing cells are derived from neural crest progenitor cells, a transient, migratory, multipotent precursor cell population known to generate much of the peripheral nervous system, epidermal pigment cells and a variety of mesectodermal derivatives (LaBonne and Bronner-Fraser, 1998). It is now well established that normal development of the thymus is dependent on correct development and patterning of neural crest cells (Bockman and Kirby, 1984; Kuratani and Bockman, 1990a; Kuratani and Bockman, 1990b; Le Lievre and Le Douarin, 1975), which contribute to the thymic mesenchyme.

This study used an immunocytochemical approach employing a novel monoclonal antibody (Mab) against turkey chromogranin A (Proudman et al., 2003), to identify and localize CgA positive cell populations within the bursa of Fabricius. The

actual production of CgA within the thymus was further corroborated using RT-PCR detection of mRNA for CgA.

Materials and methods

Primary antibodies

Monoclonal anti-turkey hypophysial CgA antibodies were obtained as a side product during the production of Mabs against a preparation of highly purified turkey hypophysial prolactin that contained trace amounts of CgA. These anti-CgA Mabs have been extensively used for immunohistochemical analyses of turkey and chicken pituitary sections. The identity of the antigen recognized by these Mabs was established by tandem mass spectrometry *de novo* sequencing of seven tryptic peptides from a turkey pituitary protein purified by immunoaffinity chromatography (Proudman et al., 2003).

Tissue sampling and processing

White Leghorn chicks obtained from a local hatchery (Hy-line) at 1 day of age were raised in wire cages at the Texas A&M Poultry Science Center. The birds were euthanized at 1, 3, 4, 7, 8, 10, and 13 weeks of age. Thymic tissues were excised and fixed for 24 hours at room temperature in Bouin Hollande sublimate. Bouin Hollande sublimate was prepared by adding 10 mL of saturated $HgCl_2$ solution to 90 mL of Bouin Hollande solution (Proudman et al., 2003). The tissue blocks were then processed for paraffin embedding using routine laboratory protocols. Seven-μm thick tissue sections

were made with a rotary microtome MT 980 (Research & Manufacturing Co., Inc, Tuscon, AZ).

Immunohistochemical reagents

TBST is a Tris-buffered saline containing 0.1% Triton X-100, pH 7.4. Trizma base and Triton X-100 were obtained from Sigma (St. Louis, Mo). Biotinylated goat anti-mouse immunoglobulin (Ig), peroxidase-conjugated streptavidin, and FITC-conjugated streptavidin were obtained from Jackson Laboratories (West Grove, PA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM and DAB (3,3'-diaminobenzidine tetrahydrochloride) were obtained from Sigma (St. Louis, MO). Cytoseal (a xylene-based mounting medium) was obtained from Stephens Scientific (Kalamazoo, MI). Vectashield (a water soluble mounting medium) was purchased from Vector Laboratories (Burlingame, CA).

Immunohistochemical detection of CgA

Immunohistochemical staining was performed on paraffin sections of thymic tissues from 1, 3, 4, 7, 8, 10, and 13 week-old chickens. The tissue sections were dewaxed and hydrated using standard lab procedures, two incubations in xylene for 5 minutes each followed by each of the following for 5 minutes each, 95% ethanol, 70% ethanol, 50% ethanol, and dH_2O, followed by 2 incubations in Lugol's reagent, 5% sodium thiosulfate, and water for two minutes each. The sections were first incubated in a moist chamber for 1 hour with 10% (v/v) normal goat serum, and then overnight with

monoclonal mouse anti-turkey CgA mab designated PLIG7F11 at a 1:3000 dilution (starting from undiluted ascitic fluid) in TBST (rinsing and dilution buffer). The next day, the sections were rinsed with TBST and the detection protocol employed a 30-minute incubation with biotinylated goat anti-mouse IgG (1:900) followed by a 30-minute incubation with peroxidase-conjugated streptavidin (1.5 μg/ml). The enzyme reaction was developed for approximately 10 minutes using 25 mg of DAB and 75 μl of 30% (v/v) H_2O_2 in 200 ml of 50 mM Tris-HCl, pH 7.4. A number of sections were counterstained with hematoxylin-eosin. The sections were dehydrated, coverslipped with Cytoseal, and immunoreactive cells were observed with an Olympus BX50 light microscope (Leeds Instruments, Inc., Irving, TX) and photographed with a Spot 110 digital camera (Diagnostic Instruments, Inc., St. Sterling Heights, MI).

RNA extraction and RT-PCR procedure

The presence of mRNA specific for CgA was verified by RT-PCR and the identity of the obtained amplicon was confirmed by nucleotide sequencing. RNA extraction was performed with the Qiagen RNeasy mini kit (Qiagen, Valencia, CA) using the standard animal tissue protocol provided by the manufacturer. Reverse transcription was carried out using the Qiagen Omniscript Reverse Transcriptase kit (Qiagen, Valencia, CA) using either random hexamers or the gene-specific downstream primer. Primers were designed based on the cDNA sequence information of a chicken hypophysial EST (gi:15083311) and purchased from Sigma Genosys (The Woodlands, TX). The primers were chosen as follows: upstream primer (CgA2US)

5’-TGAATAAAGGGGACACTAAGG-3’ and downstream primer (CgA2DS) 5’-AGCTCAGCCAGGGATG-3’. The PCR protocol was carried out using QIAGEN HotStarTaq DNA Polymerase according to the kit instructions (Qiagen, Valencia, CA). The PCR protocol used 30 cycles consisting of 30 seconds denaturation at 94 ^{o}C, 30 seconds annealing at 55 ^{o}C, and 1 minute extension at 72 ^{o}C, with a final extension at 72 ^{o}C for 10 minutes. The results from the RT-PCR were analyzed by gel electrophoresis on a 2% agarose gel analyzed using ethidium bromide fluorescence for visualization. The amplicon band was excised and purified using the QIAquick Gel Extraction Kit according to the instructions of the kit (Qiagen, Valencia, CA) and sequenced by the Gene Technologies Lab at Texas A&M University.

Results

Immunocytochemical demonstration of chromogranin A (CgA)-positive cells

Intensely stained CgA^{+} cells were readily detectable in the chicken thymus at all ages tested (Figure 2 A-D). The overall staining characteristics of the cells varied little with age, with the exception that cells seemed to increase in number with age (results not shown). Groups of immunopositive cells were distributed in a typical ring-like fashion, one ring in every thymic lobule. Counterstaining with hematoxylin-eosin (Figure 2C) showed that the ring of CgA^{+} cells was located at the medullary side of the cortico-medullary border. As a consequence, CgA^{+} cells were located in close proximity to the arterioles and the venules that provide the blood supply to the thymus. Upon high power

magnification (Figures 2B and 2D), it became clear that the CgA^+ cells constitute a population of cells with diverse morphologies. While the majority of cells display a simple round to oval shape, some cells possess very conspicuous extensions, reminiscent of a neuron-like morphology.

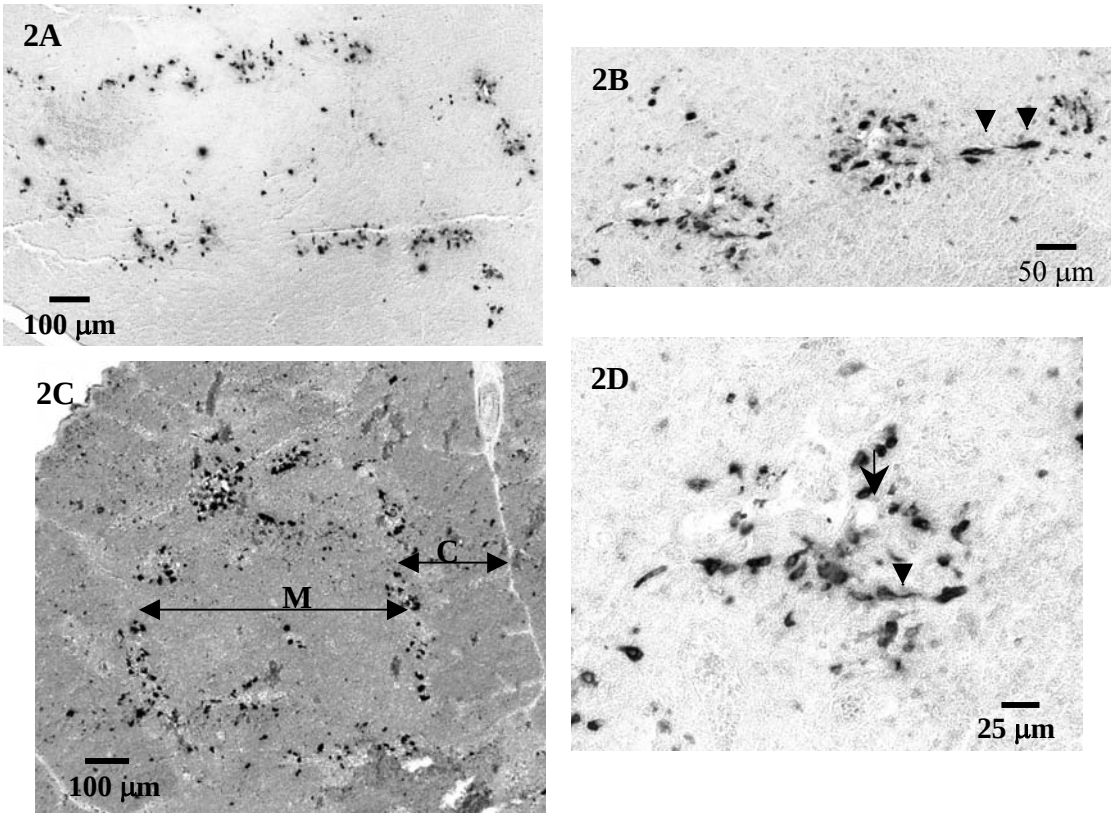

Figure 2: Immunocytochemical demonstration of chromogranin A in the thymus of a 2-week old chicken.
A. Low power magnification shows intensely stained groups of immunopositive cells that are arranged in a typical ring-like distribution. There is little evolution of this picture with age, except for an apparent increase in the number and stainability of the cells.
B. Same as A. but at higher power magnification. This picture clearly shows the morphological heterogeneity of the CgA-positive cell population. Some cells have a simple, round or oval appearance, while other cells clearly have several extensions, as indicated by the arrowheads.
C. Same as A., but with hematoxylin-eosin counterstaining. The outer region (cortex, indicated by C) of each thymic lobule is stained somewhat darker by the counterstain than the inner area (medulla, indicated by M). The ring of CgA-positive cells is located at the medullary side of the corticomedullary border, in the neighborhood of arterioles and veins that are responsible for the blood supply to each of the lobules. Both the cortex and the central area of the medulla are essentially devoid of stained cells.
D. Higher power amplification of the left hand cluster of cells from C. This photomicrograph shows another illustration of the stellate appearance of some of the cells (arrowhead). Also note the proximity of the blood vessels near the CgA-positive cluster of cells indicated by arrow.

Detection and analysis of CgA-specific mRNA

In order to confirm the specificity of the immunohistochemical data and to assess the putative production of CgA within the thymus, thymic mRNA was isolated, reverse transcribed and the cDNA was amplified with a primer set spanning a 330 nucleotide region of a chicken neuroendocrine EST with high homology to known CgA sequences (gi:15083311). As shown in figure 3, an amplicon of approximately this size was revealed upon agarose electrophoresis. This band was carefully excised and its sequence was compared with that of a number of mammalian and one amphibian homologs (Table 1), showing a high degree of sequence conservation. When compared to the gene structure of mouse chromogranin A (Wu et al., 1991), the amplified segment spans introns 3 and 4 of the CgA gene, ruling out amplification of contaminating genomic DNA. The molecular and immumohistochemical data confirm the localization and production of a substance that is highly related to CgA in the chicken thymus.

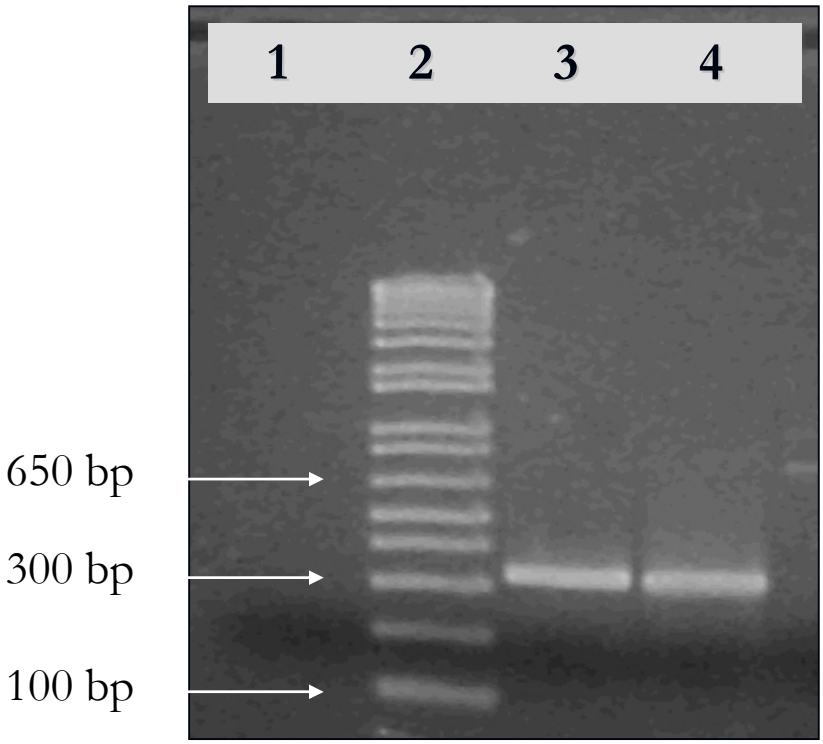

Figure 3: RT-PCR detection of mRNA for CgA in the chicken thymus.
Lane 1 = negative control; lane 2 = bp ladder; lane 3 = amplicon obtained from thymus RNA; lane 4 = amplicon obtained from hypophysial mRNA.

Table 1: Sequence alignment results of ovoinhibitor amplicon. Sequences aligned using Clustal W from the European Bioinformatics Institute, www.ebi.ac.uk/clustalw/.

```
Rattus          GGAGGTCATCTCCGACTCGCTGTCCAAACCCAGCCCCATGCCTGTCAGCCCTGAGTGTCT
Mus             GGAAGTCATCTCCGACTCGCTGTCCAAACCCAGCCCCATGCCTGTCAGCCCTGAGTGTCT
HUMCHRA         TGAGGTCATCTCCGACACACTTTCCAAGCCCAGCCCCATGCCTGTCAGCCAGGAATGTTT
Bos             CGAGGTCATCTCTGACACACTCTCCAAGCCCAGCCCCATGCCAGTCAGCAAGGAGTGTTT
Rana            TGAAGTGATTTCTGACACGCTGTCTAAACCCAACCCGGTCCCCATCACTCAGGACTGCCT
Chicken         --------------------------------------------------TGAGGAATGCCT
                                                                   ** **  *

Rattus          GGAGACCCTCCAAGGAGATGAGAGGGTCCTCTCCATCCTTCGACACCAGAATTTGCTGAA
Mus             GGAGACCCTCCAAGGAGACGAGAGGATCCTCTCTATCCTGCGACACCAGAATCTGCTGAA
HUMCHRA         TGAGACACTCCGAGGAGATGAACGGATCCTTTCCATTCTGAGACATCAGAATTTACTGAA
Bos             TGAGACACTCCGAGGAGATGAACGGATCCTCTCAATCCTGCGACATCAGAATTTGCTGAA
Rana            GGAGACTCTCCGAGGAGATGAAAGGATTATCTCCATCCTCCGGCATCAGAATCTACTGAA
Chicken         AGAGACACTCAGAGGAGATGAACGAATCATTTCTATCCTTCGCCACCAAAATTTACTGAA
                 ***** ***~ ******~**  *~~*~ * ** **~** ~* ** **~***~* *****

Rattus          GGAACTTCAAGACCTGGCGCTTCAAGGTGCCAAGGAGCGGGCCCAGCAGC----------
Mus             GGAACTTCAAGACCTGGCTCTCCAAGGTGCCAAGGAGCGGGCCCAGCAGCCGCTGAAGCA
HUMCHRA         GGAGCTCCAAGACCTCGCTCTCCAAGGCGCCAAGGAGAGGGCACATCAGC----------
Bos             AGAGCTCCAAGACCTCGCTCTCCAAGGAGCCAAGGAGCGGACACATCAGC----------
Rana            GGAGCTCCAGGAATTGGCTGCCCAAGGGGCCATGGAGAGGCTGCAGAAAG----------
Chicken         GGAACTTCAGGAAATTGCGGCTCAAGGTGCCAATGAGAGAACTCAGCAGC----------
                ~** ** ** **  * **    ***** ****~~*** *~   **  *~

Rattus          ----------------AGCAGCAGCAGCAGCAGCAGCAGCAGCAACAACAACAACA
Mus             GCAGCAGCCGCCGAAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGGAGCAGCAACA
HUMCHRA         ------------------------------------------------AGAAGAAACA
Bos             ------------------------------------------------AGAAGAAGCA
Rana            ------------------------------------------------CGAAGAAGAA
Chicken         ------------------------------------------------AAAAGAAAAA
                                                                  *~ *  *

Rattus          CAGCAGCTTTGAGGATGAACTCTCGGAAGTATTTGAGAACCAGAGCCCTGCAGCCAAGCA
Mus             CAGCAGCTTTGAGGATGAACTCTCAGAAGTGTTTGAGAACCAGAGCCCTGATGCCAAGCA
HUMCHRA         CAGCGGTTTTGAAGATGAACTCTCAGAGGTTCTTGAGAACCAGAGCAGCCAGGCCGAGCT
Bos             CAGCAGTTACGAGGATGAACTCTCAGAGGTGCTTGAGAAGCCGAACGACCAGGCCGAGCC
Rana            TCGCGGCTATGAGGAAGAGCTTTCCGGCGTCCTTGAGAAGCAGGATGACAAAGCGCCCGT
Chicken         CAGTGGCTTT--------------------------------------------------
                ~~*~* *
```

Legend: chromogranin A mRNA sequences
Rattus: *Rattus norvegicus* (Rat) GenBank: 11527393
Mus: *Mus musculus* (Mouse) GenBank: 6680931
HUMCHRA *Homo Sapiens* (Human) GenBank: 180526
Bos *Bos Taurus* (Bovine) GenBank:1890671
Rana *Rana ridibunda* (Laughing frog) GenBank: 5052372
Chicken *Gallus domesticus* (chicken) sequence as determined in the present study

* = perfect matches in homology, ~ = high homology

Discussion

This investigation reports a systematic survey of the neuroendocrine component of the avian thymus by use of traditional markers for neuroendocrine, nervous and neural crest-derived tissue. Although the presence of neuropeptides (such as neurotensin and somatostatin) in the chicken thymus was reported as early as 1978 (Sundler et al., 1978), a methodical analysis of neurosecretory cells, regardless of the secretory product involved, has to our knowledge, not been described. This study was facilitated by the availability of a new monoclonal antibody against turkey chromogranin A, whose characteristics have been reported elsewhere (Proudman et al., 2003). The obtained immunohistochemical evidence, in combination with the identification of thymic CgA-specific mRNA by RT-PCR, has allowed us to demonstrate for the first time the storage and production of chromogranin A by a discrete cell population in the chicken thymus. The identification of neuroendocrine dense-cored vesicles in the avian thymus is an exciting finding because it opens a novel avenue toward the isolation and characterization of a variety of locally produced and secreted amine and peptide hormones and neurotransmitters. This claim is justified in view of a number of reports describing other neuroendocrine molecules present in the avian thymus, such as neurotensin (Atoji et al., 1997a; Atoji et al., 1996; Atoji et al., 1997b; Carraway and Bhatnagar, 1980; Sundler et al., 1978), neuropeptide Y (Atoji et al., 1997a), substance P and vasoactive intestinal polypeptide (Atoji et al., 1997a; Gulati et al., 1997), pro-opiomelanocortin derivatives (Ottaviani et al., 1997), calcitonin gene-related peptide (Gulati et al., 1997) and somatostatin (Sundler et al., 1978); (Aguila et al., 1991).

Several of these neuropeptides have been reported to be co-stored with CgA in other tissues, such as in the chicken ultimobranchial gland (Kameda, 1991), the esophagus (Salvi et al., 1998) and in human phaeochromocytomas (Moreno et al., 1999).

Both the location of the CgA^+ cells (in the medulla of the lobules near the corticomedullary border) and their morphological heterogeneity (round to oval, but also stellate cells) reflect what has been seen previously with the nitrergic cells that have been described in the thymic microenvironment of the chicken by Gulati *et al.* (Gulati et al., 1993; Gulati et al., 1995; Gulati et al., 1998; Gulati et al., 1997). In addition, the substantial number of CgA^+ cells and the ease with which they can be stained using a moderately sensitive, non-amplified enzymatic method is noteworthy. The profile of every lobule was characterized by a ring of clustered CgA^+ cells at the medullary side of the corticomedullary border, suggesting that in a 3-dimensional view, the medullae of the thymic lobules are surrounded by a spherical lattice of neuroendocrine cells. The cortico-medullary boundaries of the chicken are important cross-roads of communication because they have been shown to receive vagal cholinergic (acetyl cholinesterase-positive) nerves, but they are also innervated by sympathetic perivascular catecholaminergic plexuses (Bulloch, 1988) that surround the arterioles from which the capillaries that serve the cortex are derived (Jeurissen et al., 1994a). As such, the CgA-positive neuro-endocrine cells are potential targets of the autonomous nervous system and of systemically circulating signals.

In conclusion, a CgA positive cell population is added to the sparse knowledge of neuroendocrine components of the avian thymus. The nature of the messenger

molecules co-stored with CgA, the mechanism by which they are released and their effects on the proliferation and differentiation of the developing T-cell populations will be topics of further investigation.

CHAPTER III

THE CHICKEN PITUITARY EXPRESSES AN OVOINHIBITOR-LIKE PROTEIN IN SUBPOPULATIONS OF SOME, BUT NOT ALL, HORMONE PRODUCING CELL TYPES*

Introduction

Ovoinhibitor, a protease-inhibiting protein, was originally purified from egg whites as a side product of partially purified chicken ovomucoid more than half a century ago (Lineweaver, 1947) and was first identified and isolated by Matsushima (Parham, 2000). It is a 50 kDa multi-domain serine protease inhibitor (serpin) belonging to the Kazal family that specifically inhibits serine proteinases such as trypsin, chymotrypsin, subtilisin and porcine elastase (Matsuda et al., 1983; Saxena and Tayyab, 1997; Shechter et al., 1977; Vered et al., 1981; Zahnley, 1975) – although none of these are likely to be the true target enzyme of ovoinhibitor in its physiological context. Immunologically, ovoinhibitor is related to the α2-proteinase inhibitor present in the serum (Barrett, 1974; Laskowski and Kato, 1980) and to vitelloinhibitor, a protease

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inhibitor recently purified from the yolk of hen's ovarian follicles (Sugimoto et al., 1996). From a functional point of view, the proteinase inhibitors in egg whites - which also include ovomucoid, ovostatin, cystatin and ovalbumin, in addition to ovoinhibitor – have been suggested to be involved in protecting eggs from microbial infection (Stevens, 1996) and in the differentiation of embryonic tissues, but these assumptions remain to be proven (Saxena and Tayyab, 1997). Serpins are the major protease inhibitors found in plasma of higher animals, where they regulate very diverse processes, including blood coagulation, fibrinolysis and inflammation (Patston and Gettins, 1994; Potempa et al., 1994; Vassalli et al., 1991). Serpins have also been described in endocrine tissues and in the brain. Examples include kallistatin, expressed in the adrenal gland (Ecke et al., 1992) and Endopin I and II, expressed in the adrenal medulla, the pancreas and the pituitary (Hwang et al., 1994; Hwang et al., 1995; Hwang et al., 1999a).

Ovoinhibitor has been demonstrated previously in the chicken oviduct and liver, where its expression is hormonally regulated by estrogens and progesterone (Stevens, 1991; Zhu et al., 2001). In addition, we have recently demonstrated the expression of ovoinhibitor in a distinct cell population in the bursa of Fabricius, the central immune organ that is the site of B-cell proliferation and differentiation in birds. A monoclonal antibody produced upon immunization with a highly purified protein preparation isolated from the bursa of Fabricius, appeared to recognize chicken ovoinhibitor, as assessed by immunoaffinity chromatography of bursal protein extracts and sequence analysis of the purified protein (Moore et al., 2004). In the present report we describe the identification of an ovoinhibitor-like isoform in the chicken pituitary.

Materials and methods

Vibratome sections

Pituitaries from laying hens of various ages were fixed overnight in phosphate-buffered 4% (w/v) paraformaldehyde (Sigma, St. Louis, MO) at 4 ºC and then transferred to a 0.5% (w/v) paraformaldehyde solution in 0.15 M phosphate buffer at pH 7.2, with 0.1% thimerosal (Sigma, St. Louis, MO) for prolonged storage at 4 ºC. Prior to sectioning, tissues were embedded in 20% (w/v) gelatin in 0.15 M phosphate buffer containing 0.1% thimerosal. Once solidified, 30-µm thick vibratome sections were made from the gelatin blocks and stored in Netwells (Costar, Cambridge, MA) in phosphate-buffered 0.5% (w/v) paraformaldehyde solution at 4 ºC until used for immunostaining.

Primary antibodies

In order to allow for the use of differentially labeled isotype-specific secondary antibodies in an immunofluorescent dual staining format, the mouse anti-ovoinhibitor monoclonal antibody (Moore et al., 2004) was combined with a panel of anti-hormone antisera produced in rabbits. Rabbit anti-chicken luteinizing hormone (LH) (Proudman et al., 1999; Puebla-Osorio et al., 2002) and rabbit anti-chicken growth hormone (GH) (Aramburo et al., 1991; Montiel et al., 1992) were validated previously in immunochemical studies in the chicken pituitary. Rabbit anti-chicken N-terminal pro-opiomelanocortin (POMC) and rabbit anti-ovine prolactin (PRL) were validated by

comparing their immunocytochemical characteristics to those of the respective monoclonal antibodies validated previously (Berghman et al., 1998; Berghman et al., 1992; Gerets et al., 2000).

Dual immunofluorescence

Costar Netwells and twelve-well plates (Costar, Cambridge, MA) were used, allowing the floating sections to be easily moved from one reagent to the next. The sections were first rinsed with Tris-buffered saline containing 0.1% Triton X-100 (TBST) and then incubated with normal goat serum at a 1:10 dilution for one hour. Next, the mouse primary antibody, undiluted ovoinhibitor hybridoma culture supernatant designated 3C8E10, and the respective rabbit polyclonal primary antisera at the appropriate dilution were applied simultaneously for overnight incubation.

The next morning, the sections were rinsed with TBST, and incubated with biotinylated goat anti-rabbit IgG (Jackson, West Grove, PA; dilution 1:500 in TBST) and rhodamine red-conjugated goat anti-mouse Ig (Jackson, West Grove, PA; dilution 1:300 in TBST) for one hour. Finally, the sections were rinsed with TBST, and incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (Jackson, West Grove, PA; 3μg/ml dilution in TBST) for 30 minutes. The sections were then rinsed, mounted on a glass slide and cover-slipped with Vectashield (Vector, Burlingame, CA) prior to microscopic examination. Olympus BX50 light microscope (Leeds Instruments, Inc., Irving, TX) was used for visualization and photographed with a Spot 110 digital camera (Diagnostic Instruments, Inc., St. Sterling Heights, MI).

Enzymatic tissue staining

Parafin embedded, 4% (w/v) paraformaldehyde fixed tissue sections of various age birds were made using a microtome. Tissues were dewaxed and rehydrated two incubations in xylene for 5 minutes each followed by each of the following for 5 minutes each, 95% ethanol, 70% ethanol, 50% ethanol, and dH_2O. Followed by 2 incubations in Lugol's reagent, 5% sodium thiosulfate, and water for two minutes each. The tissue sections with Tris-buffered saline containing 0.1% Triton-X (TBST) followed by incubation with normal goat serum (Sigma, 1:10 dilution in TBST) for 1 hour. Next, the tissue sections were then incubated overnight with a dilution of the ovoinhibitor Mab (Moore et al., 2004). The slides were rinsed the next morning using TBST. For the enzymatic staining, the sections were incubated with horse radish peroxidase conjugated goat-anti mouse Ig (Jackson, West Grove, PA) at a dilution of 1:500 in TBST for 1 hour. The sections were rinsed with PBS four times 5 minutes followed by a 5 minute incubation in TRIS solution. A 25 mg concentration DAB (Sigma) and 75 µl of 30% H_2O_2 were added to 200 ml of Tris for enzymatic color detection. The reaction was stopped by transferring the sections to dH_2O. Coverslips were applied using cryoseal XYL (Stephens Scientific, Kalamazoo, MI) and viewed using bright field light microscopy.

Reverse transcription and polymerase chain reaction

Pituitaries were collected from white leghorn hens of various ages and RNA extraction was performed using Qiagen RNeasy Kit (Qiagen, Valencia, CA). Primers

for the amplification of ovoinhibitor cDNA were selected based on Genbank sequence AH002465. The sequences were 5'- AGGATGGCAGGACTTTGG-3', for the upstream primer and OI-DS 5'- GTGCAGATGGGAGAGACT-3' for the downstream primer. As shown in Table 1, the upstream primer matches the sequence of exon 7 and the downstream primer is designed to anneal within exon 15. Reverse transcription was performed using the Omniscript Reverse Transcriptase Kit (Qiagen, Valencia, CA). The reaction mix was heated to 37^{o}C for 60 minutes, to 93^{o}C for 5 minutes and then rapidly cooled on ice. PCR reactions were performed using the HotStart Taq DNA polymerase kit (Qiagen, Valencia, CA) with an initial denaturation step at 95^{o}C for 15 minutes, and 35 cycles including denaturation at 94^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds, and extension at 72^{o}C for one minute. This was followed by a final extension step at 72 ^{o}C for 10 minutes. PCR products were separated on a 2% agarose gel, stained with ethidium bromide and analyzed under UV light. The PCR product was purified using the Qiaquick Gel Extraction Kit and sequenced using the ABI PRISM BigDye Primer Cycle Sequencing Kit.

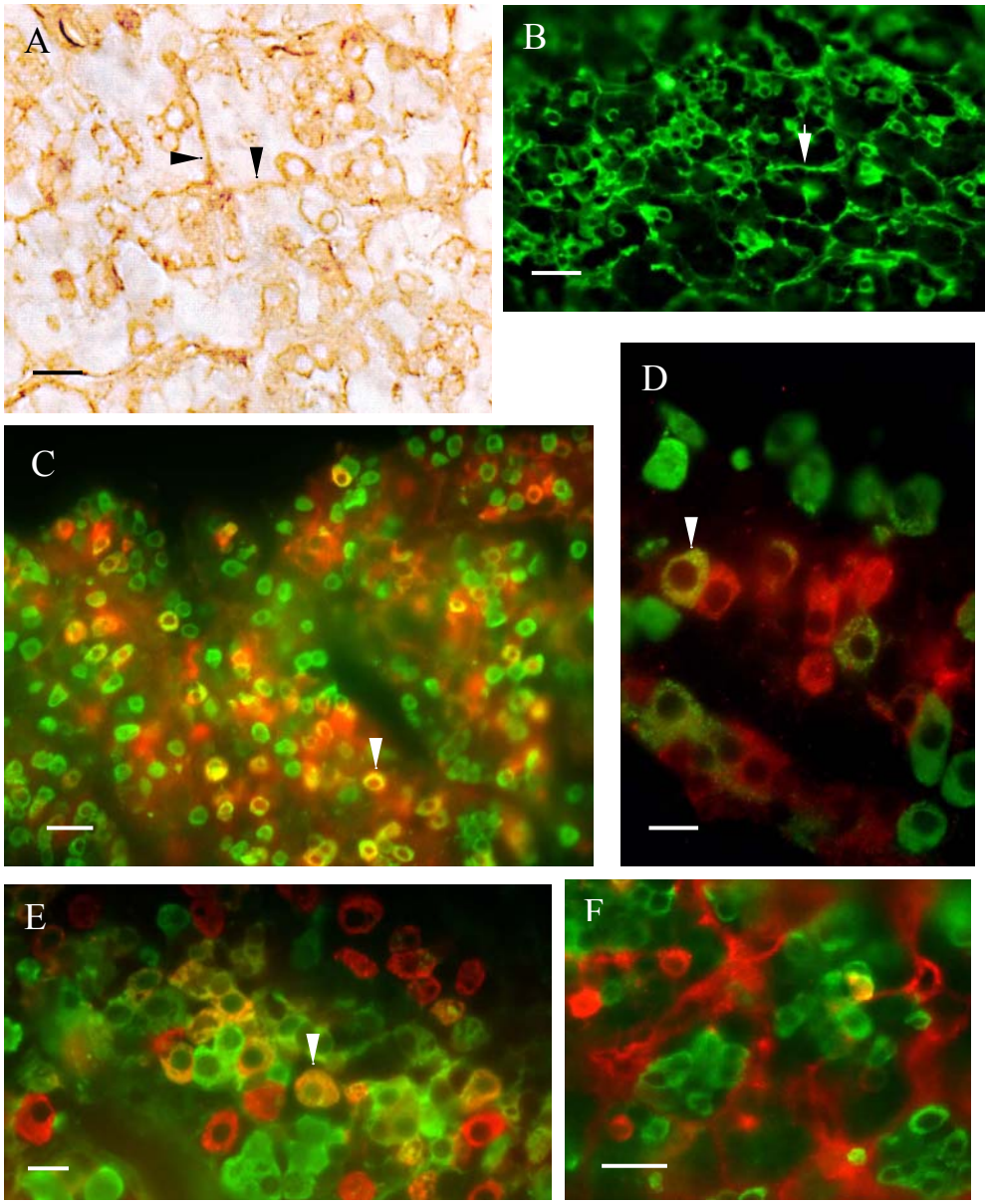

Figure 4: Immunocytochemical staining of the chicken pituitary using DAB and fluorescent immune staining.
A. Single immunoperoxidase staining with the mouse anti-ovoinhibitor monoclonal antibody. In addition to a typical cytoplasmic staining pattern, some interstitial staining can be observed as well (arrowheads). Micrometer bar = 12 μm.
B. Single immunofluorescent staining with the mouse anti-ovoinhibitor monoclonal antibody. As noted previously, in addition to a typical cytoplasmic staining pattern, some interstitial staining can be observed as well (arrow). Micrometer bar = 25 μm.
C. Dual staining mouse anti-ovoinhibitor (red) and rabbit anti-growth hormone (green). Dual stained cells can be observed (arrow head), but the majority of cells are either single green or single red stained cells. Micrometer bar = 25 μm.
D. Double staining with mouse anti-ovoinhibitor (red) and rabbit anti-luteinizing hormone (LH; green). Although most cells are single-stained, occasionally dual stained cells can be observed (arrow head). Micrometer bar = 10 μm.
E. Dual staining staining with mouse anti-ovoinhibitor (red) and rabbit anti-pro-opiomelanocortin (POMC; green). Dual stained cells indicated with an arrow head. Of all the hormone-producing cells, the corticotropes display the most coexistence with the hypophysial variant of ovoinhibitor. Micrometer bar = 10 μm.
F. Dual staining with mouse anti-ovoinhibitor (red) and rabbit anti-prolactin (green). Only single stained red or green cells could be observed; no dual stained cells were reliably identified. Micrometer bar = 16 μm.

Results

Immunocytochemistry

The overall immunohistochemical picture produced with the anti-ovoinhibitor monoclonal antibody revealed cytoplasmic staining of cells dispersed throughout both the caudal and the cephalic lobes of the adenohypophysis. Superimposed on this classic staining pattern appeared a lattice-like, interstitial immunopositive network, suggesting that the elements of the extracellular matrix are reacting with the primary antibody (Figures 4A and B). Dual immunofluorescent staining experiments showed that the staining pattern is not coinciding with any of the hormone-secreting cell populations tested (Figures 4C-F). Partial overlap was shown with cells staining positive for GH (Figure 4C), LH (Figure 4D) and POMC (Figure 4E). Cells double-stained for ovoinhibitor and LH or GH were fairly scarce. The most substantial overlap was clearly shown with POMC. No co-existence could be reliably demonstrated with PRL (Figure 4F).

RT-PCR and sequencing

As an independent control for the expression of an ovoinhibitor-like molecule in the chicken pituitary, hypophysial RNA was extracted, reverse-transcribed and amplified by use of a pair of gene-specific primers based on the published nucleotide sequence for chicken ovoinhibitor (Genbank sequence AH002465). The position of these primers is illustrated in Table 2. The results of agarose electrophoresis followed by ethidium bromide visualization showed a single amplicon with a size in the range of 800 basepairs slightly smaller than the expected size of 1000 base pairs (Figure 5).

The amplicon was then purified and sequenced. Its sequence, aligned with the nucleotide sequence of ovoinhibitor, is shown in Table 2. The obtained sequence data shows segments of perfect alignment with exon 9 through 12, with the exception that only the middle portion of each of those exons is present in the message for hypophysial ovoinhibitor variant; each exon was truncated at both the 5' and 3' ends.

Table 2: Alignment of the hypophysial ovoinhibitor variant with the published ovoinhibitor sequence.

```
Pit. Ovoinh.       ----------------------------------------------------------------------------------------------------
AH002465           AGAATGCAAGCTGGAGATCGGCTCGGTAAGTGTAACCTTATTTTGGCAGGTTGACTGCAGTAAGTACCCATCCACAGTCTCTAAGGATGGCAGGACTTTG 700
                                                  Exon 7

Pit. Ovoinh.       ----------------------------------------------------------------------------------------------------
AH002465           GTAGCCTGCCCAAGGATCCTGAGCCCGGTTTGCGGCACCGATGGTTTCACCTATGACAACGAATGCGGGATCTGCGCCCACAATGCGTAAGTGCTGCCTC 800
                                                                                                              Ex8

Pit. Ovoinh.       ----------------------------------------------------------------------------------------------------
AH002465           CCTCTTTCAGAGAGCAGAGGACCCATGTCAGCAAGAAGCATGATGGAAAATGCAGGCAGGAGATTCCTGAAGTGAGTATACACTACTTTTTGCAGATTGA 900
                                                                                                  EXON 9

Pit. Ovoinh.       ---------------------AAAAACCACTGGTGGCAAACTCCTGGTGCGCTGCCCAAGGATTCT---CCCAGTCTGTGGCACAGACGGATTTACTTAA 76
AH002465           CTGTGATCAATACCCAACAAGAAAAACCACTGGTGGCAAACTCCTGGTGCGCTGCCCAAGGATTCTGCTCCCAGTCTGTGGCACAGACGGATTTACTTA- 999
                                           ******************************************   ******************************

Pit. Ovoinh.       TGACAACGAGTGTGGCATTTGTGCCCATAATGC-----------------------ACAACATGGGACTGAGGTTAAGAAGAGCCACGATGGAAGATGC 152
AH002465           TGACAACGAGTGTGGCATTTGTGCCCATAATGCGTAAGTAACTGCTTTCCCTTTCAGACAACATGGGACTGAGGTTAAGAAGAGCCACGATGGAAGATGC 1099
                   ********************************          EXON 10      ****************************************

Pit. Ovoinh.       AAGGAGCGGAGCACCCCG----------------------CTTGACTGCACCCAATACCTGAGCAATACCCAAAACGGNGAAGCCATTACCGCCTGCC 228
AH002465           AAGGAGCGGAGCACCCCGGTAAGTGGGGAGTTTCTCCCCAGCTTGACTGCACCCAATACCTGAGCAATACCCAAAACGGTGAAGCCATTACCGCCTGCC 1199
                   ******************          EXON 11      ************************************ ******************

Pit. Ovoinh.       CCTTCATCCTGCANGAGGTCTGTGGCACTGACGGCATCACCTACAGCAACGACTGTTCTCTGTGTGCCCACAACAT----------------------- 304
AH002465           CCTTCATCCTGCAGGAGGTCTGTGGCACTGACGGCGTCACCTACAGCAACGACTGTTCTCTGTGTGCCCACAACATGTAAGCCCTGCCTCTCTCTTCCAG 1299
                   ************* ******************** *************** ***********************          EXON 12

Pit. Ovoinh.       TGAATTGGGAACCAGCGTTGCCAAAAAGCAC--------------------------------------------------------------------
AH002465           TGAATTGGGAACCAGCGTTGCCAAAAAGCACGATGGGAGGTGCAGAGAGGAGGTTCCTGAGGTAAGCGATAACTTTGTTTCCTAGCTTGACTGCAGCAAG 1399
                   ******************************                                          EXON 13

Pit. Ovoinh.       ----------------------------------------------------------------------------------------------------
AH002465           TACAAAACCTCCACGCTGAAGGATGGCAGACAGGTGGTGGCCTGCACCATGATCTACGATCCCGTCTGTGCTACCAATGGTGTCACCTATGCCAGCGAAT 1499


Pit. Ovoinh.       ----------------------------------------------------------------------------------------------------
AH002465           GCACGCTGTGCGCTCACAACCTGTAAGTACTCACTCCTTCCTCCAGGGAGCAGCGGACCAATCTTGGCAAGAGAAAGAATGGAAGATGTGAAGAGGATAT 1599
                                             Exon 14

Pit. Ovoinh.       ----------------------------------------------------------------------------------------------------
AH002465           AACAAAGGTGAGTGTGAACTTGTGTGTGCAGGAACATTGCCGTGAGTTCCAGAAAGTCTCTCCCATCTGCACCATGGAATACGTACCCCACTGTGGCTCT 1699
                             Exon 15
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Sequence alignment obtained with CLUSTAL W (1.82) multiple sequence alignment: http://www.ebi.ac.uk/servicestmp/204267.94326.aln. AH002465 represents the sequence of ovoinhibitor as published by Scott et al. (1987). Shaded boxes are the primers used for RT-PCR.
Underlined nucleotides represent the first codon of the respective exons. * = identical residues.

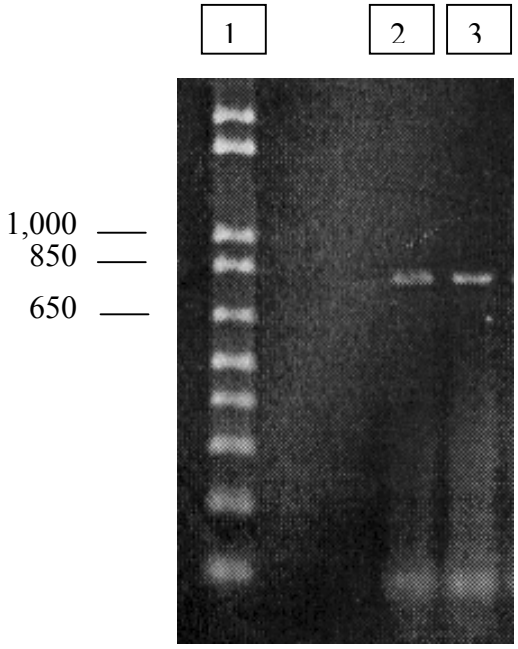


Figure 5: RT-PCR of pituitary mRNA for ovoinhibitor. The amplicon obtained from chicken pituitary cDNA is approximately 800 nucleotides in length.
Lane 1: Gibco 1 Kb Plus DNA ladder; lane 2 & 3: amplicon from chicken pituitary cDNA using the primers as indicated in Table 1.

Discussion

In the present study, an ovoinhibitor-like protein was identified in the chicken pituitary. Its cellular distribution is unlike that of any other compound described in the pituitary so far. First, the distribution was not strictly associated with that of a hypophysial hormone, and as far as an overlap existed (shown for LH, GH and POMC), only a minority of the cells of any particular hormonal cell population were dual-labeled with both the anti-hormone antiserum and the anti-ovoinhibitor monoclonal antibody. Second, an interstitial type of staining was superimposed on the cytoplasmic staining pattern, suggesting the labeling of elements of the extracellular matrix.

The cellular distribution pattern of the currently described hypophysial ovoinhibitor variant is somewhat similar to what has been described for chromogranin A in the chicken pituitary, where only a subpopulation of gonadotropes and somatotropes (depending on the age) were chromogranin A positive (Proudman et al., 2003). However, in the latter study, a majority of the cells were dual stained, while in the present study a small minority is dual-labeled. At the subcellular level, the labeling appeared to be exclusively of cytoplasmic nature, without any noticeable nuclear staining. This is in line with the recently described localization of the rat serine protease inhibitor neuroserpin in densely cored secretory granules in pituitary and adrenal cells (Hill et al., 2002). The extracellularly located ovoinhibitor-like immunoreactive material compares to that of the human tumor suppressor maspin (mammary serpin), an

intracellular serpin that is also present extracellularly, i.e. at the cell surface of human mammary epithelial cells (Pemberton et al., 1997).

The combination of immunocytochemical data and nucleotide sequence undeniably indicate a strong structural relationship between the previously described ovoinhibitor molecule (Scott et al., 1987) and the presently reported hypophysial ovoinhibitor variant. Nevertheless, it is also obvious that the two molecules are not identical. First, the amplicon obtained from hypophysial mRNA is about 20% smaller than predicted from the previous reports of the ovoinhibitor sequence in the chicken oviduct (800 nucleotides instead of the expected 1000 nucleotides) (Scott et al., 1987). In addition, the obtained cDNA sequence shows up- and downstream truncation of each of the represented exons. Alternative splicing of serpin genes has been reported as a parsimonious way to generate different inhibitors with varying target specificity (Kruger et al., 2002).

The biological significance of serpins is not always clear, because in many cases the corresponding serine protease has not been identified. Exceptions include the rat pituitary serpin raPIT5a, which forms an SDS-stable complex with granzyme B suggesting a role for this inhibitor in modulation of apoptosis (Hill et al., 1998). Another rat serpin has been reported to interact with tissue plasminogen activator (Hill et al., 2002). Plasminogen activators are expressed in many tissues, including endocrine tissues (Danglot et al., 1986). Plasmin has recently been found to process chromogranin A into bioactive peptides in chromaffin cells (Jiang et al., 2001). Given the discrepancy between the hypophysial ovoinhibitor variant and the previously described ovoinhibitor

structure, however, it cannot be ruled out that the former might no longer function as a protease inhibitor, since serpins have been also reported to be potentially active as hormone carriers or play roles in the control of cell signaling (Kruger et al., 2002). Further studies, including the complete structural characterization of this molecule, will be needed to shed light on its biological significance for the function of the pituitary, but the presence of ovoinhibitor in both immune and endocrine tissues, provides a clear link between the interactions of the neuroendocrine and immune systems.

CHAPTER IV

CHARACTERIZATION OF THE BURSAL SECRETORY DENDRITIC-LIKE CELL POPULATION OF THE BURSA OF FABRICIUS USING VARIOUS IDENTIFICATION TECHNIQUES

Introduction

The immune system is a vital defense system of an organism against the outside world. The Bursa of Fabricius is a unique organ of humoral immunity in birds, allowing for B-cell development and proliferation (Masteller et al., 1997) that does not exist as an anatomically defined organ in mammalian species. Bursal secretory dendritic-like cells (BSDC) were first identified in 1978 by Olah and Glick, and have since been deemed an important part of the bursal microenvironment with a potential role in B-cell selection and differentiation (Glick, 1978; Glick and Olah, 1987b; Glick and Olah, 1993a; Glick and Olah, 1993b). Under electron microscopy, these cells have one or more cell extensions, with granules located in one process, giving a polarized appearance, see Figure 6 (Olah and Glick, 1987). It has been previously shown that antibodies against vimentin (Dako, clone 3B4) can be used to stain BSDCs in immunohistochemically (Olah et al., 1992a; Olah et al., 1992b). Vimentin is an intermediate filament protein

that is found in cells that originated from mesenchymal tissue (Carey and Zehner, 1995) and has been localized in the thymus (Minko and Olah, 1996) and spleen (Olah and Glick, 1994) of the chicken. While immunhistochemical identification of the BSDCs with vimetin is possible, the putative secretory product and the exact relationship between the secretory cells and the lymphopoietic cells involved in bursal follicle formation were unknown in 1993 (Glick and Olah, 1993a; Glick and Olah, 1993b) and have yet to be discovered. Previous studies have estimated the BSDC cell population to be 0.5% of the total bursal population (Olah and Glick, 1987). In culture, 80% of the B-cell population dies within 6 hours of removal from the bursal cellular architecture (Compton and Waldrip, 1998; Neiman et al., 1994). This provides a vital method for enrichment of the stromal cell population in culture, increasing the concentration of the BSDC cell population approximately 10-fold.

The current study focuses on the further characterization of the BSDC cell population using several methods including, immune (antibody-mediated) staining, flow cytometry, scanning electron microscopy and laser capture microdissection for localization and characterization of the BSDC population.

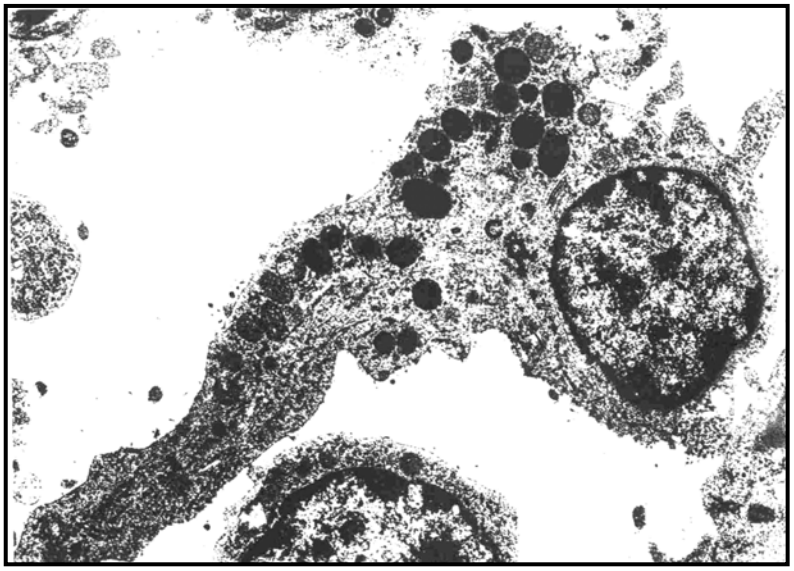


Figure 6: Electron microscope image of the bursal secretory dendritic-like cell. The nucleus is off-centered and there are visible granules below the surface on the cellular extension of the cell (Olah and Glick, 1987).

Materials and methods

Immunohistochemistry on tissue sections

Paraffin embedded, 4% (w/v) paraformaldehyde fixed bursal tissue sections of birds between 1 day and 7 weeks of age, were made using a rotary microtome (RMC MT 980). Tissues were dewaxed and rehydrated by two incubations in xylene for 5 minutes followed by each of the following for 5 minutes each, 95% ethanol, 70% ethanol, 50% ethanol, and dH_2O.

Enzymatic staining procedures began by rinsing the tissue sections with Tris-buffered saline containing 0.1% Triton-X (TBST) followed by incubation with normal goat serum (Sigma, St. Louis, MO; 1:10 dilution in TBST) for 1 hour. The tissue

sections were then incubated overnight with the primary antibody at the appropriate dilution in TBST; anti-ovoinhibitor antibody (Moore, 2004, ascites fluid) at a 1:9000 dilution, anti-vimentin Mab (Dako Cytomation, Clone Vim 3B4 Carpinteria, CA) at a 1:150 dilution, Mab 1A4 (Dr. Tom Scott, Clemson University) hybridoma supernatant used undiluted and, CVI-ChNL-74.3 (Dr. S. Jeurissen from the Central Veterinary Institute, Netherlands) (Jeurissen et al., 1992) at a dilution of 1:150. The slides were rinsed the next morning using TBST and incubated with horseradish peroxidase-conjugated goat-anti mouse Ig (Jackson, West Grove, PA) at a dilution of 1:500 in TBST for 1 hour. The sections were rinsed with PBS four times 5 minutes followed by a 5 minute incubation in a 50 mM Tris-HCl buffer (pH 7.4, no Triton). Diaminobenzidine (DAB; Sigma) at 25 mg and 75 µl of 30% H_2O_2 were added to 200 ml of Tris-HCl buffer for enzymatic color detection. The reaction was stopped by transferring the sections to dH_2O. Coverslips were applied using cryoseal XYL (Stephens Scientific, Kalamazoo, MI) and viewed using bright field light microscopy (Olympus BX50 light microscope; Leeds Instruments, Inc., Irving, TX) and photographed with a Spot 110 digital camera (Diagnostic Instruments, Inc., St. Sterling Heights, MI).

Immunofluorscence staining procedures began by rinsing the tissue sections with Tris-buffered saline containing 0.1% Triton-X (TBST) followed by incubation with normal goat serum (Sigma, 1:10 dilution in TBST) for 1 hour. The tissue sections were then incubated overnight with the appropriate dilution of a primary antibody, including the TrkB receptor polyclonal antibody (Pab) (Santa Cruz Biotechnologies, Santa Cruz, CA at a 1:200 dilution) and anti-vimentin. The slides were rinsed the next morning

using TBST. The slides were then incubated with either biotinylated goat anti-mouse IgG or biotinylated goat anti-rabbit Ig antibodies (Jackson Immunoresearch Labs, West Grove, PA) at a 1:500 dilution for 1 hour. The tissues sections were rinsed with TBST and fluorescently labeled. The tissues sections were rinsed with TBST and fluorescently labeled with FITC-conjugated streptavidin. (Jackson, West Grove, PA at a 1:500 dilution). To reduce quenching, Vectashield solution (Vector laboratories, Burlingame, CA) was placed on the stained tissue sections, followed by a coverslip.

Bursal cell culture and enrichment

Bursal tissue was removed from day-old male white leghorn chicks (Hyline Bryan, TX). Tissue was digested by three, two-hour incubations with 2μg/ml collagenase (Invitrogen, Carlsbad, CA) at 37^{o}C while shaking. Cells were collected and either used directly for flow cytometric staining or scanning electron microscopy procedure, or prepared for enrichment procedures

To obtain an adherent cell population, aliquots of bursal single cell suspension from collagenase digested bursa were placed in T75 (Becton Dickson, Menlo Park, CA) vented flasks overnight for enrichment of the cell population. The following morning the B-cells and debris were rinsed away using sterile PBS. The resulting adherent cells were used for scanning electron microscopy. The adherent cell population is devoid of B-cell and all other cells that do not attach during the overnight incubation.

The non-B-cell population, was enriched by an overnight incubation while shaking to prevent cell attachment. The next day the cells were concentrated by

histopaque solution (Histopaque-1077, Sigma, St. Louis, MO) removing red blood cells, the dead cells and debris. During the histopaque procedure,15 mls of overnight bursal cell culture was layered on top of 15 mls of histopaque solution and was centrifuged for 30 minutes at 400 x g. A gradient was formed by centrifugation and the second cell layer was collected and rinsed in sterile PBS. Once collected, the enriched cell population was washed in PBS (Sigma,St. Louis, MO) and used for flow cytometry and scanning electron microscopy.

Flow cytometry

All flow cytometry experiments were preformed in collaboration with Dr. Roger Smith of the Veterinary Pathobiology Department at Texas A&M University. The flow cytometry procedure was performed on both total bursal single cell culture suspensions as well as an enriched adherent cell sample. The cell samples were washed using sterile PBS solution and centrifugation for 10 minutes at 4^{o}C at 1100 rpm. The anti-vimentin antibody required intracellular staining, so the cells were fixed and permeabilized prior to staining using the BD Cytofix/Cytoperm kit (BD Bisosciences, San Diego, CA). Upon fixation any potential Fc receptors or other non-antigen-specific immunoglobulin binding sites on any of the cells were blocked with goat IgG (Sigma) for 30 minutes followed by staining with the primary antibodies used. For vimentin staining, the cells were incubated with anti-vimentin antibody (Dako, 1:150 dilution) for 1 hour. Mouse Anti-Chicken MHC II-R-PE and Anti-Chicken IgG-FITC (SouthernBiotech Birmingham, AL) were titrated using various dilutions (ranging from 1:50 to 1:600

dilutions in PBS) for optimal signal-to-noise ratio of the cell labeling and incubated for 1 hour prior to sorting analysis. For staining with the, Mab 1A4 (From Dr. T. Scott), and CVI-ChNL-74.3 (from Dr. S. Jeurissen) antibodies, the cells were incubated with the antibody at the proper dilution (Scott undiluted and Jeurissen 1:150) in PBS followed by secondary antibody incubation, FITC-conjugated F(ab)'2 goat anti-mouse IgG(Jackson ImmunoResearch, West Grove, PA). Unstained cells and cells incubated only with secondary antibodies were used as controls.

Once the cells were labeled, a cell sorting analysis was performed using the FACSCalibur (Becton Dickenson, San Jose, CA) and the labeled cell populations were analyzed based on percentage labeled cells relative to the total cell counts.

Laser capture microdissection

Bursal tissue samples were collected from 6-week old broiler chickens, snap frozen by dry ice and held at -80^{o}C until sectioning. Tissue sections 8 μm in thickness were made using a cryostat and were post-fixed in acetone for five mintues. After the sections were air dried, they were immersed in buffer and the staining process was initiated. Hematoxylin and Eosin stained cryosections were used for laser collection (Shandon, Pittsburgh, PA). The Arcturus PixCell II Laser Capture Microdissection System was set on smallest diameter (7.5 mm) and the intensely stained cortico-medullary border of the bursal follical was collected on CapSure LCM caps (Arcturus Bioscience Inc, Mountain View, CA). Total RNA was extracted from the microdissected samples using the Qiagen RNeasy Kit (Qiagen, Valencia, CA) and RT-

PCR was performed using the Qiagen RT-PCR kit (Valencia, CA). Ovoinhibitor-specific primers were used as previously described in chapter III. Amplicons were verified by ethidium bromide gel electrophoresis.

Scanning electron microscopy

All scanning electron microscopy experiments were preformed in collaboration with Dr. Robert Droleskey of the USDA, College Station, TX. Total bursal cell samples and enriched cell samples were used for scanning electron microscopy. Eight-well chamber slides treated with CC2, a proprietary coating for increased cell attachment were used. Total bursal cell suspensions were made using the described collagenase procedure, and placed overnight in coated 8-chamber slides (Nunc Lab-Tek II-CC2, VWR, West Chester, PA). The following day the chambers were rinsed with sterile PBS to remove dead cells and debris and then growth media (RPMI 1640, Sigma) containing 5 μg/ml of LPS from *Salmonella enterica* serovar Typhimurium was added to separate chambers for 5 minutes, 3 hours; controls consisted of cells that were only exposed to growth media without LPS.

The cells were fixed by adding the fixative containing a 2% gluteraldehyde solution in 100mM phosphate buffer containing 100 mM sucrose (pH = 7.4) directly to the cells in the chambers. The slides were incubated at 4^{o}C until further processing. While still in the chamber slides, the samples underwent post-fixation in 1% osmium tetroxide, followed by 1% uranyl acetate dissolved in phosphate buffer. Following fixation, the chambers were removed from the slides and dehydrated by increasing

concentrations of ethanol up to 100%. After dehydration, cells were critical point dried using CO_2. The resulting dried slide was then scribed and separated with a diamond pen along the gasket lines of each chamber. Each separated well was mounted on stubs and coated with gold followed by examination by scanning electron microscopy (Cambridge Stereoscan 200, Cambridge Instruments, Deerfield, IL).

Results

Immunohistochemical staining

The immunohistochemical staining indicates the populations stained by the antibodies used. Staining with the anti-ovoinhibitor Mab revealed labeled cells in both the cortex and medulla, with the most intense staining in the cortico-medullary border of the bursal follicle (Figure 7A). Upon closer inspection the stained cells have an oval to ellipsoid appearance (Figure 7B).

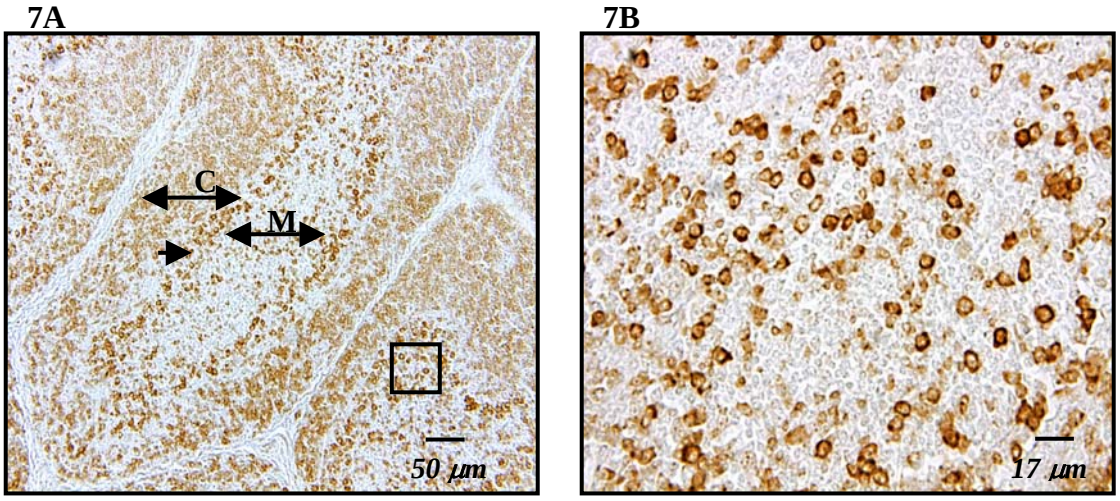


Figure 7: Immunohistochemical staining of the bursa of Fabricius using a monoclonal antibody against the ovoinhibitor protein.
A. An enzymatic staining of a bursal follicle, the cortex (C) and medulla (M) are sporadically stained, with the most intense staining occurring in the cortico-medullary border, labeled with an arrow. Bar equals 50 μm, the box is an area shown at higher magnification in Figure 7B
B. An enzymatic staining of the cortico-medullary border region of a bursal follicle. Bar equals 17 μm.

The staining results of the vimentin Mab displayed a similar cellular distribution as the ovoinhibitor-positive cells with cortico-medullary staining of the bursal follicle. The cells in the cortex and medulla have more of a dendritic cellular appearance, with long cell extensions (Figure 8).

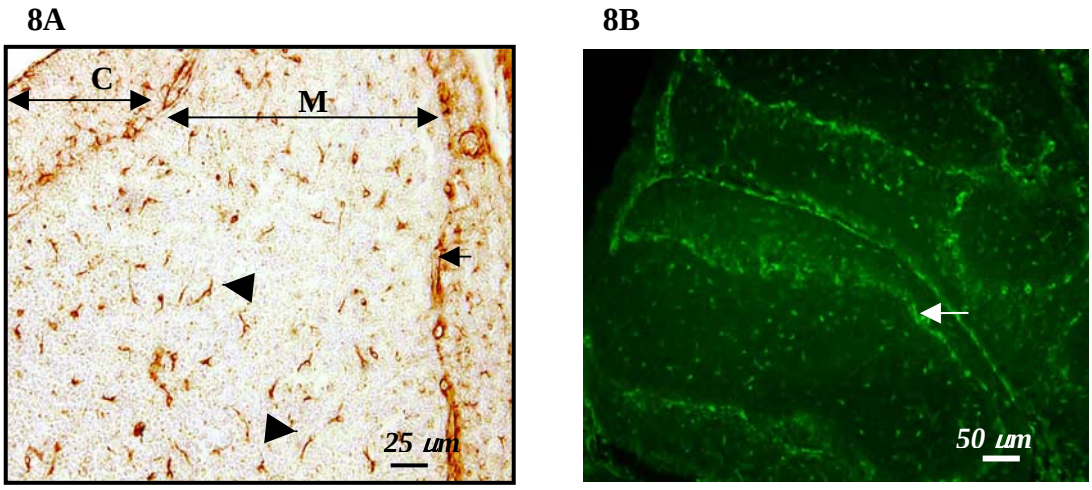


Figure 8: Immunohistochemical staining of the bursa of Fabricius using a monoclonal antibody against vimentin.
A. An enzymatic staining of a bursal follicle, the cortex (C) and medulla (M) are sporadically stained, with intense staining in the cortico-medullary border, labeled with an arrow. Note the dendritic appearance of the cells, labeled with arrowheads. Bar equals 25 μm.
B. A fluorescent antibody (FITC) staining of the bursal follicle, the arrow indicates the corticomedullary border region of a bursal follicle. Bar equals 50 μm.

Staining experiments using the Mab CVI-ChNL-74.3, show a staining located in the medullary area of the bursa of Fabricius (Figure 9A). Under higher magnification, some of the stained cells have a dendritic appearance with cellular extensions (Figure 9B).

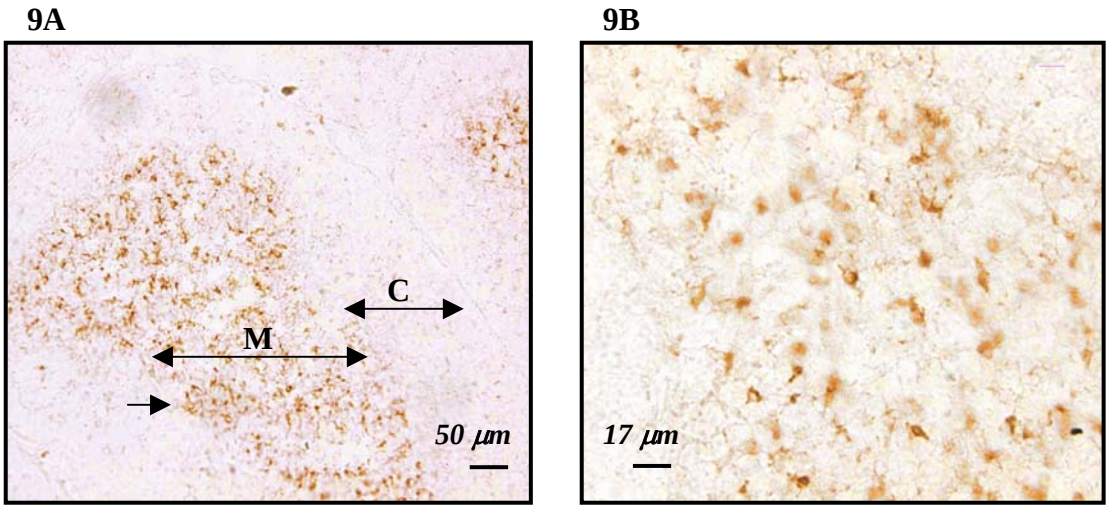


Figure 9: Immunohistochemical staining of the bursa of Fabricius using monoclonal antibody CVI-ChNL-74.3 from Dr. S. Jeurissen from the Central Veterinary Institute, Netherlands, which labels follicular dendritic cells (Jeurissen et al., 1992).
A. An enzymatic staining of a bursal follicle, the cortex (C) is stained lightly, with majority of the staining occurring in the medulla (M), light staining in the cortico-medullary border, labeled with an arrow. Bar equals 50 μm.
B. An enzymatic staining of the medullary region of the bursal follicle. Bar equals 17 μm.

The enzymatic stainings with the hybridoma supernatant from Dr. Tom Scott of Clemson University, Mab 1A4, show a staining located in the cortex of the bursa of Fabricius (Figure 10A). Under higher magnification, some of the stained cells have a dendritic appearance with cellular extensions (Figure 10B).

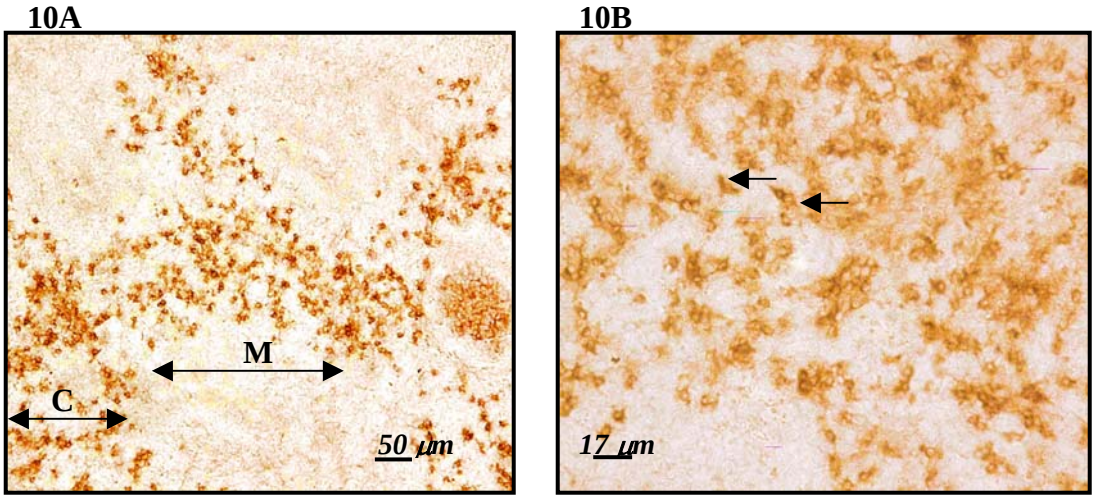


Figure 10: Immunohistochemical staining of the bursa of Fabricius using a monoclonal antibody from Dr. Tom Scott, a hybridoma supernatant (Mab 1A4) which labels dendritic-like cells.
A. An enzymatic staining of a bursal follicle, the cortex (C) is stained intensely with little staining in the medulla (M), no detectable staining in the cortico-medullary border. Bar equals 50 µm.
B. An enzymatic staining of the cortical region of the bursal follicle. Bar equals 17 µm.

The fluorescent staining experiments using anti-TrkB receptor polyclonal antibody (Pab) display a concentration of reactive cells in the cortex of the bursal follicle (Figure 11A). Using higher magnification, localization of individual stained cells is possible (Figure 11B).

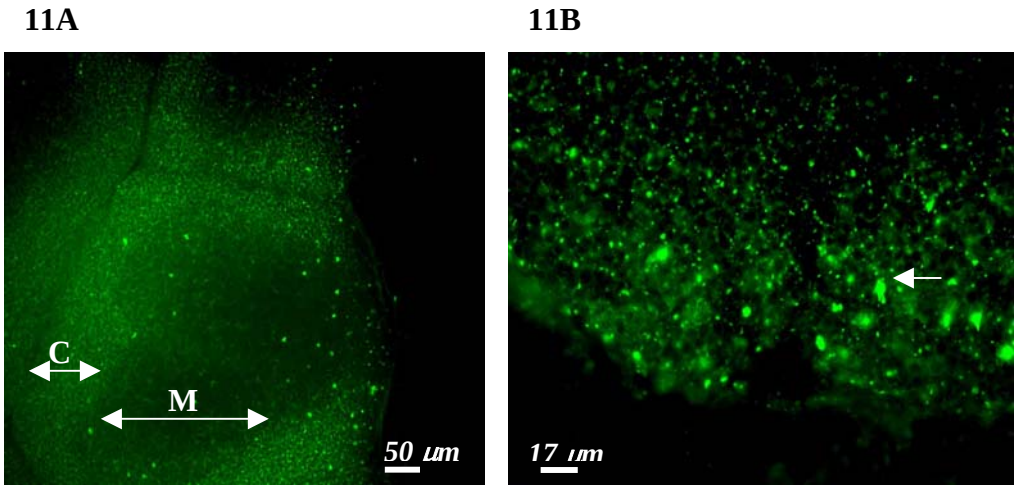


Figure 11: Immunohistochemical staining of the bursa of Fabricius using a polyclonal antibody against the neurotrophin receptor TrkB.
A. A fluorescent (FITC) staining of a bursal follicle, the cortex (C) is stained mainly with minimal staining in the medulla (M), no detectable staining in the cortico-medullary border. Bar equals 50 µm.
B. A fluorescent (FITC) staining of the cortical region of the bursal follicle, note the cell staining indicated by the arrow. Bar equals 17 µm.

Bursal cell enrichment

After overnight attachment to plastic, the bursal cell population is reduced to the adherent cell population. There are several cell types visible through light microscopy

of the attached cells. There are long rod shaped cells, flattened cells, oval cells, and cells with a more dendritic appearance (Figure 12).

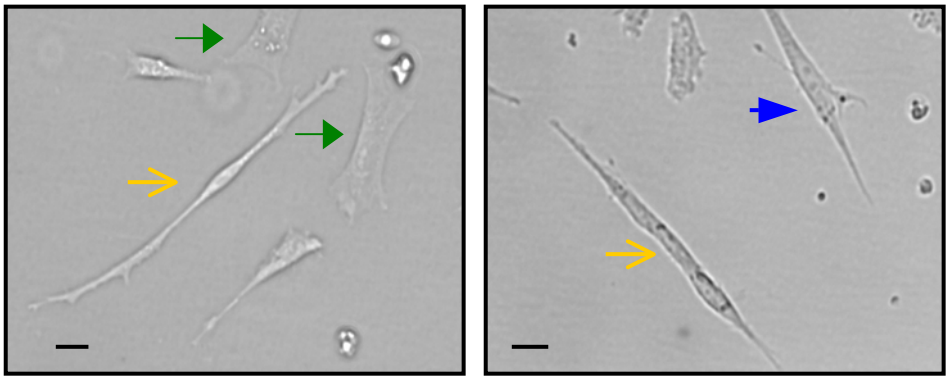


Figure 12: Light microscopy of an un-stimulated adherent cell population attached to cell culture flask. Note different morphologies indicated by colored arrows. Yellow arrows (→) indicate long rod shaped cells, the green arrow (→)indicates the flattened cells, and the blue arrow (→) indicates a dendritic like cell. Bar equals 10 µm.

Scanning electron microscopy

The adherent cell population was stimulated with LPS to determine if visible differences were seen in cell morphology caused by stimulation. The cell populations appeared to show the same cellular morphology whether they had been stimulated or not. There are several cell morphologies seen in the adherent cell population, most of which contain thin, hair-like cellular extensions projecting from their surface (Figure 13 A-D). The BSDC population is contained in this sample, but their exact morphology is indeterminate.

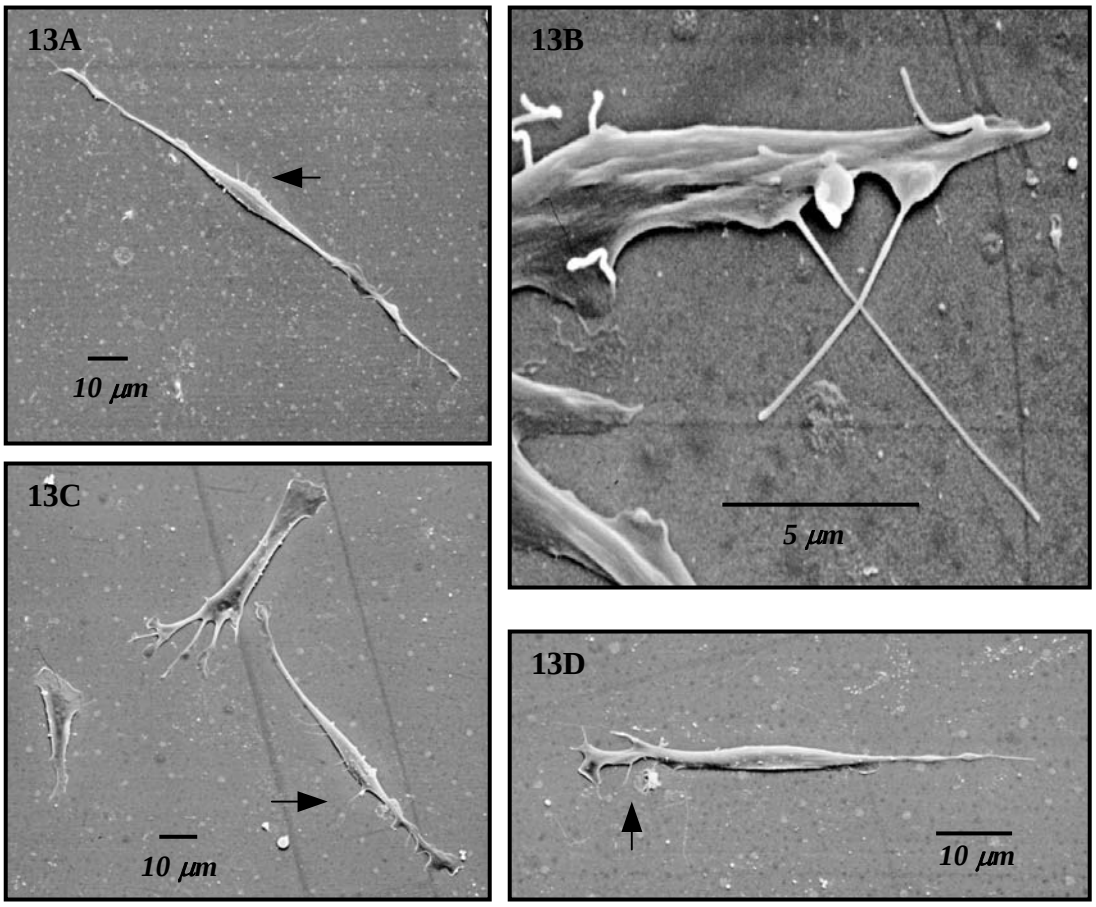


Figure 13: Scanning electron microscopy of the adherent cell populations from the bursa of Fabricius. Several cell morphologies are seen without a detectable difference between control (13A) and LPS stimulated (13 B-D) sample groups. Determination could not be made whether these are different stages of the same cell type or if they represent truly different cell populations.

A. Adherent cell from the control group, an example of the long cell thin cells found within the population. Note the thin hair-like extensions coming from the cell, indicated by the arrow. The bar represents 10 μm in length.

B. Very high magnification showing the hair-like extensions found on several of the cell types present in the adherent cell population. The bar equals 5 μm in length.

C. Representatives of three cell morphologies observed by SEM. The fine hair-like extensions are indicated by the arrow. The size bar equals 10 μm.

D. An individual cell with a long cell body with a branched end. The arrow indicates the thin hair-like extensions. The size bar equals 10 μm in length.

Flow cytometry

Total bursal cells were cultured overnight, followed by histopaque centrifugation to remove the dead B-cells, red blood cells, and debris. In initial flow cytometry experiments the antibody that recognizes vimentin, which is an intracellular staining, requiring fixation and permeabilization, was used for cell sorting. Of the total bursal population, less than one percent of the total cell number stained with the vimentin antibody (data not shown). An enriched cell population was developed to remove the B-cells (estimated to represent 80 % of all bursal cells) and increase the BSDC percentage of the population. The vimentin-positive population was increased greatly by the overnight enrichment, making up approximately 10% of the enriched population (Figure 14A). Unfortunately, due to intracellular staining, the necessary permeabilization and fixation not only killed the cells but also compromised the mRNA integrity. Several cell surface antibodies were tested for sorting analysis in order to find a suitable replacement for the vimentin Mab. Literature indicated that the BSDC population may be stained using anti-chicken IgG (aCh IgG) Mab (Jeurissen et al., 1994b; Olah and Glick, 1987; Ratcliffe, 1989). Initial flow experiments indicated potential labeling with anti-Ch IgG (Figure 14B), but under further investigation with strict controls, there were indications of non-specific binding, possible via Fc recptor binding or immune complexes (Figure

14C). The original titrations showed consistent highly stained cell populations, but after running specific controls including blocking with unlabeled anti-chicken IgG (aCh IgG) followed by phycoerythrin (R-PE)-labeled anti-Ch IgG, unfortunately, the blocked cells displayed a higher staining than the R-PE labeled aCh IgG alone. Another control cell sample was incubated with Chicken IgG followed by R-PE labeled aCh IgG which also had a higher cell staining than the R-PE labeled aCh IgG alone. In conclusion, the sorting analysis of cells indicates the labeling with aCh IgG is mostly non-specific and cannot be sorted from the specifically labeled population.

An antibody given by Dr. Tom Scott from Clemson Univeristy, Mab 1A4, labeled a large cell population, including the BSDC population, although it was not as specific as needed (Figure 14D). An antibody given by Dr. Susan Jeurissen from the Central Veterinary Institute, Netherlands, CVI-ChNL-74.3, labeled cells that could not be sorted from the negative control population incubated with the FITC-conjugated F(ab)'2, because the staining intensity was not high enough from controls (Figure 14D). In conclusion, currently, there is not a cell surface antibody available with the capabilities of sorting the BSDC population.

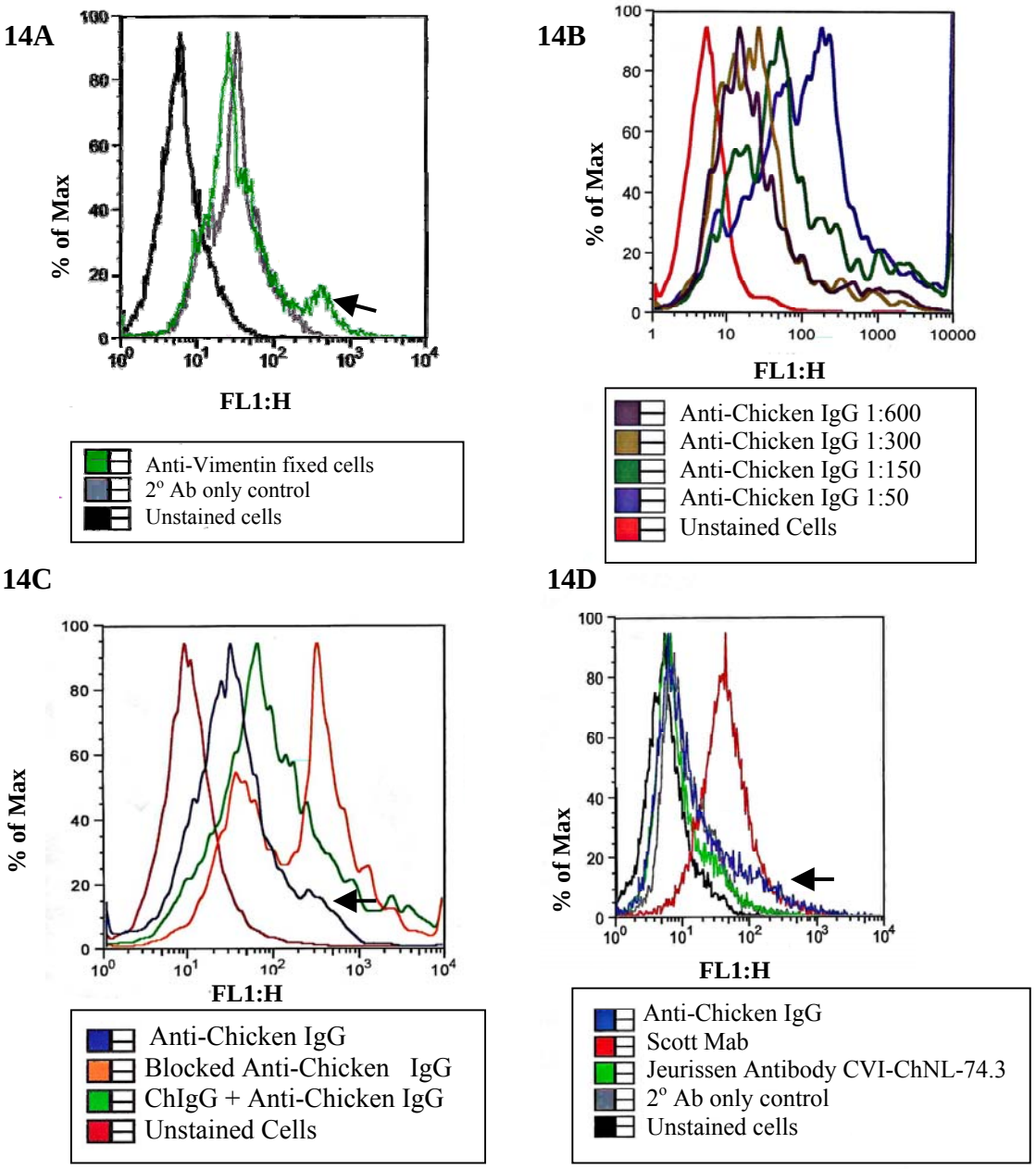


Figure 14: Flow cytometric analysis of overnight enriched cell population. The X-axis represents the number of positive cells and the Y-axis indicates the intensity of staining. A shift of the peak to the right indicates increased cell staining.
A. Sorting analysis using anti-vimentin Mab on fixed and permeabilized cells. Arrow indicates the specifically labeled population, approximately 10% of the total enriched population.
B. Antibody titration of anti-chicken IgG (aCh IgG) Mab; showing significant staining of unstained cells at all dilutions tested.
C. Sorting analysis results of aCh IgG Mab compared to various controls. Cells were blocked with unlabeled aCh IgG followed by phycoerythrin (R-PE)-labeled aCh IgG, a large increase in labeled cells was observed compared to aCh IgG only. Another control cell sample was incubated with Chicken IgG followed by R-PE-labeled aCh IgG, again note increase in labeled cell population. The labeling of cells with antiCh IgG is mostly non-specific and cannot be sorted from the specifically labeled population, indicated by an arrow.
D. Other antibodies used Mab 1A4, from Dr. T Scott, and CVI-ChNL-74.3, from Dr. S. Jeurissen. The Mab 1A4 antibody labels a large population of cells, including the potential BSDC population, but unfortunately, it cannot separate the BSDC population from the rest of the stromal cells. The CVI-ChNL-74.3 antibody does not produce a population greater than the "secondary antibody only" control.

Laser capture microdissection

Laser capture microdissection (LCM) was performed on hematoxylin and eosin stained frozen, non-fixed tissue sections. The entire cortico-medullary border area was targeted with the laser and the tissue was removed with the specially designed cap (Figure 15A). The tissue removed with the cap was used for RNA extraction and reverse transcription PCR. Amplicons were verified by agarose gel electrophoresis followed by ethidium bromide staining for visualization. Figure 15B shows that a sufficient amount of intact mRNA can be obtained by LCM in order to perform RT-PCR.

Frozen bursal tissues sections stained with the anti-vimentin Mab were used for LCM of specifically stained cells to collect the BSDC population. Due to the unusual shape of the BSDC, with their cellular extensions, collection was hindered. Several hundred cells were collected and RNA extraction followed by amplification were attempted. Several methods for extraction and amplification were performed, but the integrity of RNA was compromised due to the extended time for the staining procedure. In order to collect the requisite number of cells to attempt molecular analysis, it would require the collection of thousands of cells and countless hours and resources that were unavailable.

15A **15B**

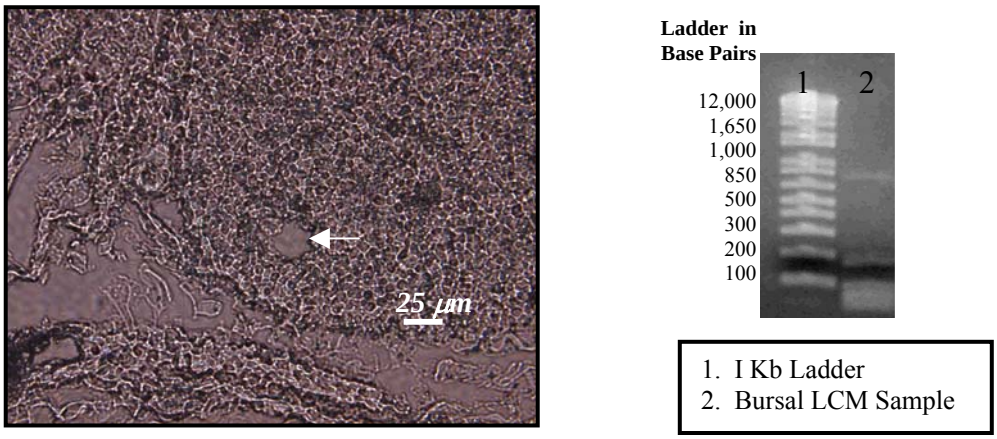


Figure 15: LCM of bursal tissue.
A. Tissue section of bursal follicle used for the collection of tissue for LCM. The arrow indicates a hole in the tissue where material was removed with the cap during the LCM procedure. The LCM removed tissue was used for RNA extraction and reverse transcription PCR.
B. The PCR products were run on a 2% agarose gel with ethidium bromide visualization. The PCR products following LCM collection of tissue samples from the cortico-medullary transition zone are located in lane 2.

Discussion

The bursa of Fabricius of the chicken is a valuable model for studying the humoral immune system, not only in birds but potentially also in mammals. The humoral immune system is a complicated system involving interactions between the B-cells and the stromal cells that surround them in the bursal follicle (Glick, 1994; Ratcliffe, 2002). The stromal cell population is made up of several cell types, including macrophages and bursal secretory dendritic-like cells (BSDC). The BSDC population is a poorly understood cell population and identification is limited to morphological appearance (stellate shape and the presence of secretory granules) and staining with an anti-vimentin Mab., Vimentin is an intermediate filament and thus an intracellular antigen (Olah et al., 1992a; Olah et al., 1992b). This study implemented a combination of several (mostly immunochemical) methodologies to gain more insight into the BSDC cell population.

Immunocytochemistry was used to identify the cell population via immunoenzymatic and fluorescent labeling methods. The most useful feature was the localization of a substantial portion the of vimentin-positive cell population along the cortico-medullary border region of the bursal follicle. The anti-ovoinhibitor antibody also stained many cells in this very region, while the immunoreactivity obtained with other the antibodies we tested was much more concentrated in either the medullary or cortical region of the follicle.

The intense staining of the cortico-medullary border was used as a guide for selecting cells for LCM removal and subsequent RNA extraction and reverse

transcription of cells removed by LCM. Although in a few cases RT-PCR has been attempted starting from LCM on immunostained sections, the duration of most immunostaining protocols, in combination with the necessity to permeabilize the cell membranes with non-ionic detergents in order to make antigens accessible, tend to compromise mRNA quality and quantity (Fend et al., 1999; Goldsworthy et al., 1999; Rekhter and Chen, 2001). After a quick histochemical staining of frozen sections with hematoxylin and eosin (which allowed the identification of the cortico-medullary border with good confidence) and removing cells along the entire cortico-medullary border, we were able to amplify ovoinhibitor sequences from the cells collected by RT-PCR. In an attempt to collect a purer sample of the BSDC population for molecular analysis, the tissue was stained with anti-vimentin Mab and several hundred positive cell profiles were collected and processed for reverse transcription PCR. The BSDC have processes that extend from the center of the cell, making it very difficult to capture the entire cell, without inadvertently acquiring parts of the surrounding cells as well. The increased time of processing for specific staining, and the shape of the cell, lead to a decreased yield of quality and purity of RNA for molecular analysis. Even after attempts to amplify the RNA using the MessageAmp procedure designed for amplification from small quantities of RNA (Ambion, Austin, TX), from several hundred collected cells, the quantity was too low for use in the molecular analysis (results not shown).

As an alternative, flow cytometry was assessed as a potential method to purify the BSDC population. Initial attempts used whole bursal single cell suspensions that were fixed, permeabilized and immunostained with the anti-vimentin Mab. The cell

sorting analysis showed the vimentin-positive population to be less than 1% of the total bursal cell population. Such a low frequency of positive cells does not allow for efficient fluorescence-activated sorting, especially because the FACS apparatus available to us, FACSCalibur (Becton Dickenson, San Jose, CA), was not built as a dedicated cell sorter (R. Smith, pers. comm.). The vimentin-positive population clearly needed to be enriched in order to allow for the use of cell sorting as a valid option. Previous studies have shown that 80% of the B-cell population dies within 6 hours of cell culture (Compton and Waldrip, 1998; Neiman et al., 1994). In order to enrich the vimentin positive cell population, the bursal single cell suspension was incubated overnight, while shaking to prevent attachment. The following day the dead B-cells, RBC and debris were removed by histopaque centrifugation and the resulting cell population was fixed, premeabilized and stained with the anti-vimentin Mab. The analysis showed that the vimentin population was increased 10-fold, to approximately 10% of the stromal (non-B-cell) population. This increase was dramatic, but the viability of the cells and the integrity of the RNA was compromised due to fixation and permeabilization procedures. A cell surface antibody was required for sorting to yield a functional cell population. Several antibodies were tested in conjunction with staining for vimentin (as the most reliable marker for BSDC) to identify a suitable cell surface antibody. The use of anti-chicken IgG looked promising initially with consistent labeling of the same cell population (Glick, 1995; Glick and Olah, 1993b). Positive staining of the BSDC for IgG would indeed be consistent with a potential function in presenting immune complexes to the neighboring B-cells in the medullary compartment (Yasuda et al., 2002).

Unfortunately, the seemingly positive cell population turned out to be an artifact due to non-specific labeling, potentially via Fc binding. When incubated with the secondary antibody alone, sorting revealed a greater population of labeled cells than with the primary and secondary. Attempts to block the signal with chicken IgG followed by staining, caused an increase in positive cells. The use of a non-labeled (cold) primary antibody, i.e. anti-chicken IgG, followed by FITC-labeled primary antibody, (i.e. FITC-conjugated anti-chicken IgG) still increased the positive signal. Also IgG antibodies from other species, such as mouse and bovine yielded increased positive cell populations (results not shown). Clearly, labeling with an anti-chicken IgG Mab was not able to specifically label and sort the BSDC cell population. The antibody provided by Dr. T. Scott did label the vimentin population, but also labeled a lot of other cells. Based on the CVI-ChNL-74.3 Mab (Jeurissen et al., 1994b) cells could not be reliably separated from unstained or "secondary antibody only" cell populations. Finally, while an antibody against the neurotrophin receptor TrkB in theory should be useful for FACS analysis and sorting, since TrkB is a cell surface receptor. Unfortunately the anti-TrkB antibody tested immunocytochemically in this study (see Figure 6), did not stain cells in the cortico-medullary border, nor in the medulla, contrary to what had been reported by Ciriaco et al. (1997), ruling out also this option for specific sorting of the BSDC. In conclusion, in order for flow cytometry to become a valid option, a specific cell surface antibody needs to be developed to allow for sorting of a pure BSDC population. At this point, we would be able to immunize with a mixture of bursal stromal cells that is estimated to contain 10 % pure BSDC (based on staining with anti-vimentin). While this

is not an ideal situation, in theory monoclonal antibody technology is able to generate BSDC-specific antibodies, on condition that these cells do express a unique cell surface molecule.

Scanning electron microscopy (SEM) gave us very high power, detailed images pictures of the enriched, adherent cell population. Not surprisingly, several distinct cell morphologies seem to be represented in the adherent cell population, the BSDCs are among the populations seen. Specific identification requires extensive SEM characterization that will require antibody labeling. The LPS-stimulated adherent cell population was not visually different from the un-stimulated population based on our SEM observations.

In conclusion, a variety of methods for the enrichment of the vimentin-positive BSDC cell population were tested. Through elimination of the apoptotic B-cells, we were able to increase the target cell concentration by 10-fold. Several methods, based on existing monoclonal antibodies, were used to try and isolate and further characterize the BSDC population of the bursa of Fabricius. The conclusion from these FACS experiments is that, in order to obtain a 100% pure cell population for *in vitro* functional and molecular analysis, a specific cell surface marker is required, and should be a focus of future experiments.

In this study, two novel approaches have been developed for the enrichment of the non-lymphoid cells of the bursa of Fabricius. One model utilizes the ability of a subset of the stromal population to adhere to plastic for enrichment. The other utilizes the death of B-cells in culture by overnight incubation followed by density

centrifugation on a Histopaque cushion to remove dead cells, red blood cells and debris, leaving the stromal population for further analysis. These strategies give a 10-fold increase in the BSDC population over the total bursal cell population and can be used for further examination, including protein and molecular analysis following stimulation with a mitogen. This research has shown that there is an urgent need for the development of a BSDC-specific cell marker antibody, and with the newly developed model systems as a guiding resource, the much needed antibody will be developed in the near future.

CHAPTER V

THE EFFECTS OF CLOACAL INOCULATION WITH *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM ON GENE EXPRESSION IN THE CHICKEN BURSA OF FABRICIUS

Introduction

The bursa of Fabricius, the site of B-cell maturation and development is unique to avian species (Glick, 1994). While the bursa is the central humoral immune organ in birds, it also functions as a Gut-Associated Lymphoid Tissue) GALT, as it is connected to the cloaca via the bursal duct. Chickens can reflux material in a retrograde manner, both from the contents of the gut and from their environment, into the lumen of the bursa (Ekino et al., 1985b). When particles and fluids are collected from the chick's environment onto the anal lips and deposited in the lumen of the bursa, the process is referred to as "cloacal drinking". The transport from the anal lips to the bursal lumen is extremely efficient and takes less than five minutes to complete, as can easily be demonstrated by depositing a drop of India ink on the anal lips of a neonatal chick. After endocytosis by the bursal follicle-associated epithelium (FAE), the material is directly transported to the medullary compartment (Naukkarinen and Sorvari, 1984) whose volume and mitotic index was reported to increase after uptake of *Bordetalla pertussis* (de Azevedo and Betti, 1993), while the cortex of the bursal follicles did not undergo any noticeable changes. The functional dichotomy between the cortical and

medullary compartments of the bursa is further supported by the fact that the cortex, which has a 2- to 3-fold higher mitotic index than the medulla (Reynolds, 1987), is the source of the 60% of peripheral B-cells that are short lived, naïve, and thus highly diverse (Paramithiotis and Ratcliffe, 1994b), while the medulla is the likely source of 35% of blood B-cells with a lifespan in the order of a few weeks (Paramithiotis, 1996) that have undergone positive selection in the bursa by interacting with environmentally derived antigens (Paramithiotis and Ratcliffe, 1994a). Indeed, bursal duct ligation in the late embryonic stage, isolating the bursa from environmental stimuli, was shown to suppress the immune response (Ekino et al., 1980). The medullary compartment is believed to be the major site for antigen-driven B-cell proliferation after hatching and *in situ* stimulation of bursa cells by environmental antigens to the bursa accelerates S phase entry rates in the medullary areas (Ekino, 1993). Furthermore, administration of foreign antigens into the bursal lumen prior to bursal duct ligation in the embryo has recently been shown to cause a significant increase in lymphocytes with a productive V-J joint in the neonatal chicken bursa compared with the isolated bursa (Arakawa et al., 2002). While it has been proposed that immune complexes formed by maternal antibodies and gut-derived antigens are presented to medullary B-cells by dendritic cells (Yasuda et al., 2002), the molecular mechanism of how the bursal medullary compartment processes environmental antigens remains largely unknown.

In this study, two questions were addressed: first, does bursal inoculation with live bacteria (*Salmonella enterica*, in this case) cause an inflammatory *i.e.* innate immune response, and second, does antigenic stimulation lead to increased survival of

proliferating B-cells, potentially by a decreased rate of apoptosis? These questions were evaluated by measuring the relative expression of genes known to be associated with the inflammatory response or with apoptosis using quantitative PCR. In order to assess the initiation of an innate immune response, the expression of interleukin-16 (IL-16), interleukin Il-12 (IL-12), interleukin-1β (IL−1β), heat shock protein 90 (Hsp90), and heat shock cognate 70 (Hsc70) were quantitated 30 minutes, 3 hours and 24 hours after cloacal inoculation with *Salmonella*. Toll like receptors (TLRs) on antigen-presenting cells recognize LPS and induce the production of pro-inflammatory cytokines, such as IL-12 (Akira et al., 2001). Heat shock proteins (HSP) Hsp90 and Hsc70 are classified as molecular chaperones, and Hsc70 functions in particular to protect cells from environmental stress (de la Rosa et al., 1998; Kang et al., 1999; Morales et al., 1998). HSP have also been shown to play a major role in the initiation of the innate immune system. Environmental stresses as well as LPS infection have been documented to increase the synthesis of heat shock proteins (Wallin et al., 2002). Hsp70 and Hsp90 perpetuate the infection signal of LPS to antigen presenting cells (APCs) via TLR-2 and CD91, respectively (Prohaszka and Fust, 2004; van Eden et al., 2003).

Potential alterations of the spontaneous rate of apoptosis were assessed by analyzing the relative gene expression of apoptosis-related genes, such as caspase 8 (Casp 8), caspase 3 (Casp 3) and Death-Associated Protein 5 (DAP5). In the caspase cascade system, Casp 8 is activated by Fas-associated death domain (FADD). After autocatalytic activation, Casp 8 induces Casp 3 activation (Barkett et al., 2001; Liu et al., 2004). DAP5 is a caspase-activated translation factor which further supports the

translation of apoptosis-related proteins (Henis-Korenblit et al., 2002; Warnakulasuriyarachchi et al., 2004). Finally, an antagonist to the cell death cascade, B-cell lymphoma 2 (Bcl-2) was included in this study (Rayet et al., 2003; Sato et al., 2002).

Materials and methods

Cloacal inoculation

Four-day old white leghorn male chicks (Hy-Line International, Bryan, TX) were challenged intra-cloacally with 0.5 ml of a 10^5 cfu/ml culture of *Salmonella enterica* serovar Typhimurium (Presque Isle Cultures, Presque Isle, PA). Four groups of three birds were inoculated with bacteria and one group was given sterile saline as the unchallenged control. Bursal tissue was collected and stored in RNAlater (Ambion, Austin, TX) at 30 minutes, 3 hours, and 24 hours post-challenge; control samples were collected at 24 hours. Three bursae from each time point were homogenized and total RNA was extracted using the Qiagen RNeasy Kit (Qiagen, Valencia, CA). Samples were analyzed in duplicate by real-time PCR (see below), yielding six data points per time point.

In vitro cell collection and stimulation

In order to discriminate between B-cells and adherent cells as the potential source of the gene shifts observed *in vivo,* adherent cells were enriched by overnight incubation of a bursal single cell suspension. The adherent cell population was

considered free of B-cells since bursal B-cells are non-adherent and quickly undergo apoptosis upon disruption of the bursal intercellular contacts (Neiman et al., 1994). The bursa of Fabricius was removed from day old white leghorn chicks (Hy-line Bryan, TX). Tissue was digested by three two-hour incubations with 2μg/ml collagenase (Invitrogen, Carlsbad, CA) at 37^{o}C while shaking. Cells were collected and placed in T75 vented flasks (Becton Dickson, Menlo Park, CA) overnight. The next day non-adherent cells (mostly dead B-cells) were removed and the flasks were rinsed with sterile PBS (Sigma, St. Louis, MO). Growth medium (RPMI 1640, Sigma) containing 5 μg/ml of LPS from *Salmonella enterica* serovar Typhimurium was added to separate flasks for 30 minutes, 3 hours or 24 hours; growth medium only was used as the control. At the appropriate time the cells were collected and RNA was extracted using the Trizol method by adding 2 ml of Trizol to each flask on ice. After a five minute incubation, the Trizol and cells were collected and frozen at 20^{o}C. Chloroform was added and the upper aqueous phase was collected. Isopropanol was added to create an RNA pellet, which was washed with ethanol and then air-dried. Once dry, 30 μl of nuclease-free H_20 was added and the sample was stored at -20^{o}C until reverse transcription.

Quantitative PCR

Gene specific primers were designed (Table 3) based on published sequences in NCBI databases available online, with the exception of the sequence for Chicken Il-12β which was a gift from Dr. James Zhu at Texas A&M University. Primer sets were designed using either Primer Express 4.1 (ABI systems, Applied biosystems) or

GeneFisher (http://bibiserv.techfak.uni-bielefeld.de/genefisher/). Primer stringency criteria included the selection of amplicon sizes of 70 to 150 base pairs and a maximum GC content of 55%. β-actin was used as a calibrator gene for calculations of gene expression (Thellin et al., 1999). Each primer sequence was verified for uniqueness by comparison with genomic sequences using BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Pro-inflammatory genes and genes related to innate immunity included IL-12β, IL-16, IL-1β, HSP90 and HSC70. Genes related to apoptosis included Casp 3, Casp 8, Bcl-2 and DAP5.

Table 3: Primer sequences designed for QPCR. IL-1β, Interleukin-1β; IL-16, Interleukin-16; IL-12β, Interleukin-12β; C3, Complement Component C3; A2HSP, Alpha-2-Heat Shock Glycoprotein; Hsp 90, Heat Shock Protein 90; Hsc 70, and Heat Shock Cognate 70; Casp 3, Caspase 3; Casp 8, Caspase 8; DAP5, Death Associated Protein 5; Bcl-2, B-cell lymphoma-2.

Gene Target	**Upstream**	**Downstream**
IL-1β	GAAGGTGCGGAATAGGA	CCGAGAAGGGATGACGA
IL-16	CTGCCACCCAGTCTTGTGAGA	ATGACACCTGGTTACTGATGGAATAG
IL-12 β	TAAAGTAGACTCCAATGGGCAAATG	AGAACGTCTTGCTTGGCTCTTTATAG
C3	GAGGAGTGCATCGCCTTCAG	CACTTCCCCATGGCAGATCTT
A2HSP	TGTCTACCTCATGCTCCTTGAGATC	GATCCTCAGGAAGCAGTTGACAA
HSP 90	GGCAGTTTGGTGTGGGTTTCT	GCTCGTCATCGTTGTGCTTG
HSC 70	TCATCAAGCACACAAGCCAGTA	AGCGAGCTCTGGTGATCGAT
β-Actin Calibrator	CTGATGGTCAGGTCATCACCATT	TACCCAAGAAAGATGGCTGGAA
Casp 3	GATGCTGCAAGTGTCAGA	ATCGCCATGGCTTAGCA
Casp 8	AGACTTCCTCTTGGGCATGACTA	TTGGCACAGTGACTGTATGTACCA
DAP5	ACAAGGACAGTCGAAGGATATGC	GCAGGTCTCAGGCTTATCTCATC
Bcl-2	TGAGCAGAGGTCACGTA	CACACTGTGGAACAGCA

Following RNA extraction, reverse transcription was performed with the RETROscript cDNA kit using oligo dT (Ambion Austin, TX). A template concentration

of 0.5 μg cDNA per tissue sample and Sybr Green PCR Master Mix (Applied BioSystems, Foster City, CA) were used in combination with the respective gene-specific primers for the PCR reaction, which consisted of initial denaturation temperature of 95^{o}C for 10 minutes followed by forty cycles of a 15 second hold at 95^{o}C then a 1 minute hold at 60^{o}C. To reduce inter-assay variability, the same amount of template per sample was used in each run, and each run included chicken β-actin for normalization purposes. Each sample was run in duplicate.

The relative expression of each of the target genes in comparison to the calibrator gene (β-actin) was calculated using the ΔΔCt method (Lehmann and Kreipe, 2001; Livak and Schmittgen, 2001); analysis of variance was performed and significant differences between means were determined by least squares mean method using SAS ($p<0.05$).

Primer specificity verification

In order to verify the length of the quantitative PCR products, the amplicons were verified separated on a 2% agarose E-gel, (Invitrogen, Carlsbad, CA) stained with ethidium bromide and analyzed under UV light.(Figure 16).

Results

Primer verification

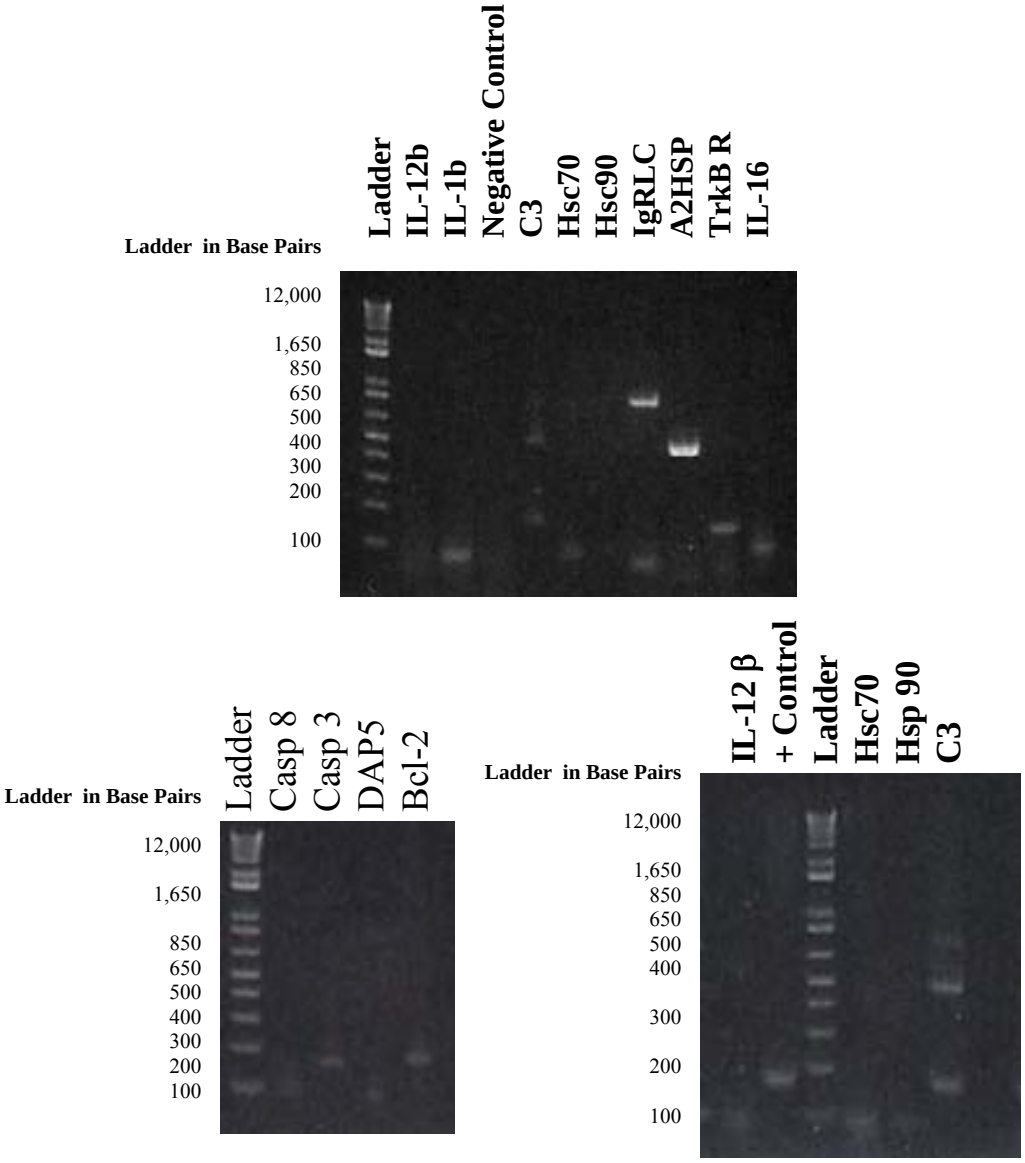


Figure 16: A 2% agarose gel stained with ethidium bromide for verification of PCR product size. The amplicons of all genes that will be further discussed were in the range from 70-200 base pairs as predicted by the primer selection criteria. The primer pairs designed and used for C3, IgRLC, and A2HSP contained bands in the expected region, but also produced bands that were greater than expected size and are not considered for further analysis.

Expression profile of genes related to innate immunity

The pro-inflammatory, innate immunity-related genes included in this study were IL-12β (p40), IL-16, IL-1β, HSP90 and HSC70. A dramatic (approximately five-fold) and sustained increase was observed in the expression of IL-12β at all time points, from as early as 30 minutes post-inoculation, up until 1 day after challenge with live *Salmonella* (Figure 17) . By contrast, all other genes related to activation of the innate immune system (including IL-16, IL-1β, Hsc70 and Hsp90) showed a moderate transient decrease (at 30 min and 3 hours post-challenge) followed by up-regulation (increase in the order of 50 to 100%) 24 hours after inoculation, although this general numerical trend was not statistically significant ($p<0.05$) at all time points for all genes (Figures 18-22). For instance, in the case of IL-1β, the transient decrease at 30 min. and 3 hours was significantly different from the increased level at 24 hours, but neither was significantly different from the level at time 0.

In order to distinguish between B-cells and macrophages as potential sources of IL-12 up-regulation (Degen et al., 2004), the adherent cells of the bursa were isolated and separated from the B-cell pool by overnight incubation of a bursal single-cell suspension in static culture. After removal of non-adherent cells, the adherent cells were exposed to LPS. As shown in Figure 18, no significant changes of IL-12β were observed after 3 hours of exposure to LPS. This seems to rule out the macrophages as the source of the up-regulation of IL-12 observed *in vivo*.

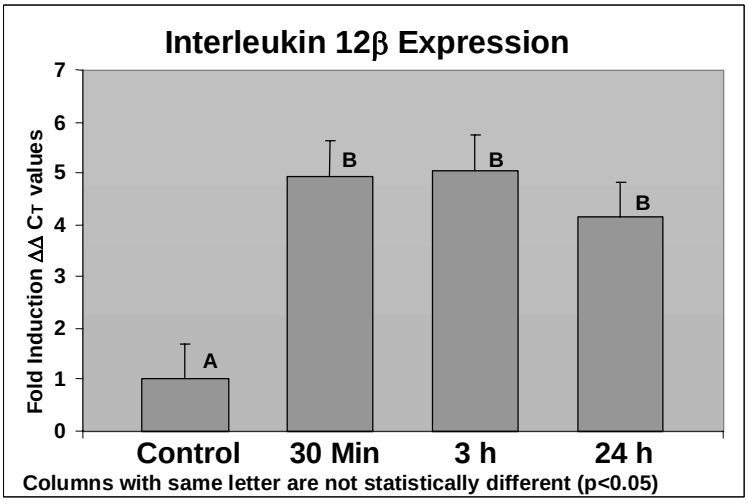


Figure 17: Interleukin-12β expression in the bursa of Fabricius after *in vivo* cloacal inoculation with *Salmonella*. Compared to controls, IL-12β gene expression displayed a fast, robust and sustained increase up until 24 hours after cloacal inoculation. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

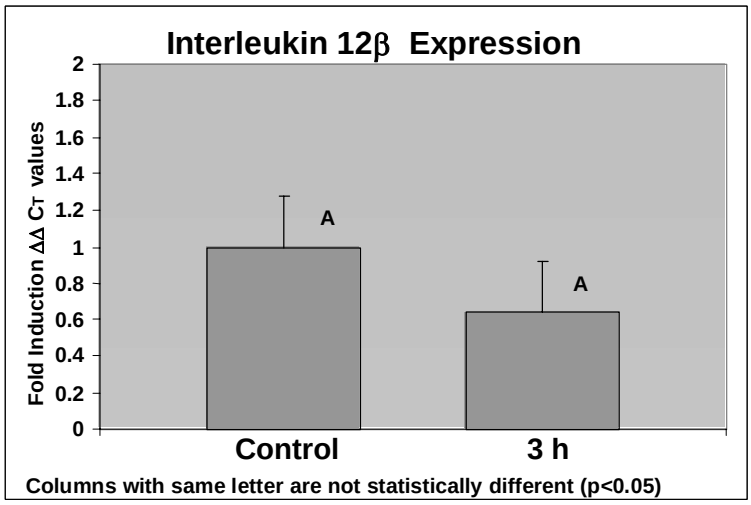


Figure 18: Interleukin-12β expression in the adherent cell fraction of the bursa of Fabricius *in vitro* after exposure to LPS (5 μg/ml) from *Salmonella*. No significant changes were observed after a 3-hour exposure. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

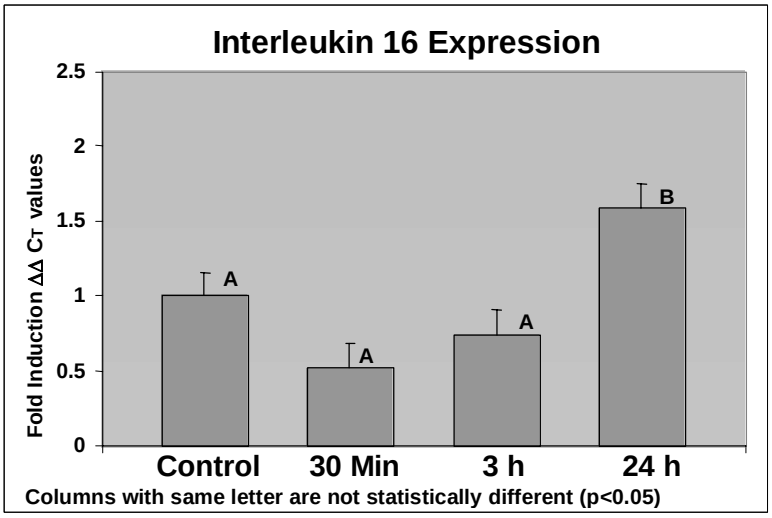


Figure 19: Relative expression of Interleukin-16 using β-actin as the calibrator gene. Twenty four hours after LPS stimulation, IL-16 gene expression is significantly increased compared control levels. The 30 minute and 3 hours gene expression of IL-16 are not significantly different from the control. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

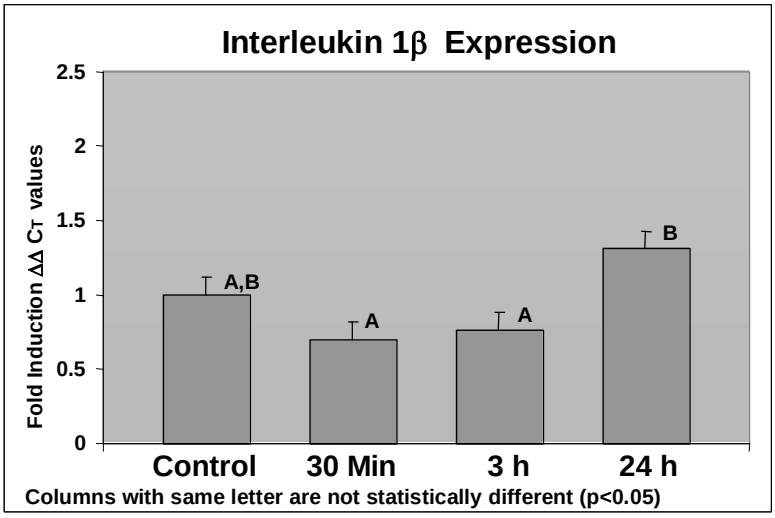


Figure 20: Relative expression of Interleukin-1β using β-actin as the calibrator gene. IL-1β gene expression values are not significantly different from the control levels at any of time points sampled. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

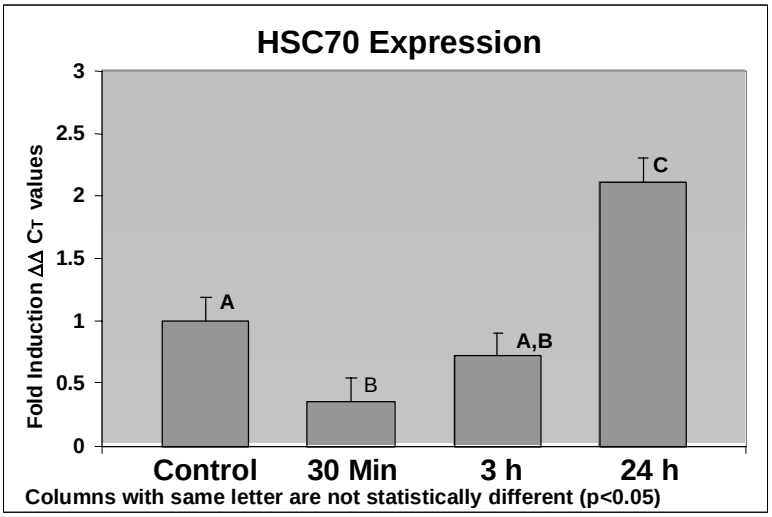


Figure 21: Relative expression of HSC70 using β-actin as the calibrator gene. The gene expression of Hsc70 decreases initially at the 30 minute time point, and increases significantly by the 24 hour time point. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

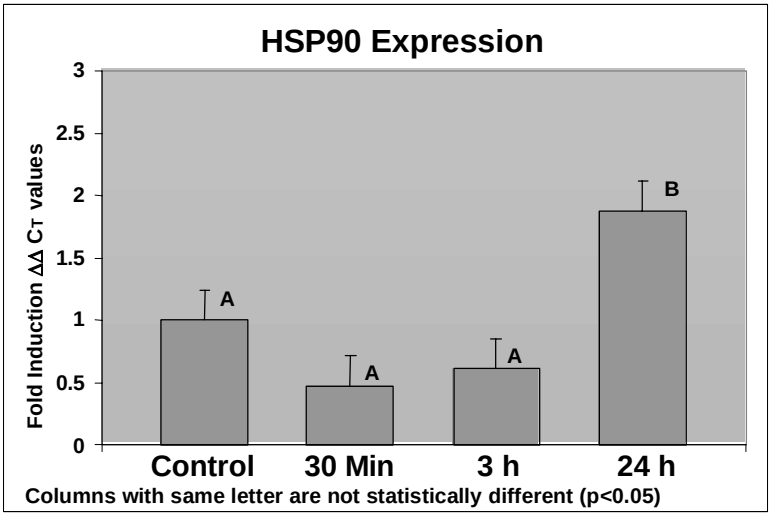


Figure 22: Relative expression of HSP90 using β-actin as the calibrator gene. The statistical analysis shows Hsp90 gene expression to be significantly increased from the control sample at the 24 hour time point. The 30 minute and 3 hours gene expression of Hsp90 were not significantly different from the control. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

Expression profile of apoptosis-related genes

The other gene set that was investigated as a function of time after cloacal inoculation with *Salmonella* were pro- and anti-apoptotic genes. Caspase 8 (an initiator caspase) gene expression increased two-fold compared to controls 30 minutes after cloacal inoculation, and returned to control levels by 3 hours post-challenge (Figure 23). By contrast, the relative gene expression of caspase 3 (an executioner caspase) was not significantly increased until 24 hours after inoculation with *Salmonella* (Figure 24). The DAP5 gene expression initially decreased significantly, as compared to the control sample at the 30 minute time point, and returned gradually to control levels at the twenty-four hour time points (Figure 25).

Upon bursal challenge with *Salmonella*, the expression of the anti-apoptotic gene Bcl-2 (B-Cell Lymphoma 2) changed only marginally during the first 3 hours (although it was slightly up-regulated after 30 minutes), but was dramatically (i.e. approximately twelve-fold) decreased 24 hours post-inoculation *versus* the saline inoculated control (Figure 26).

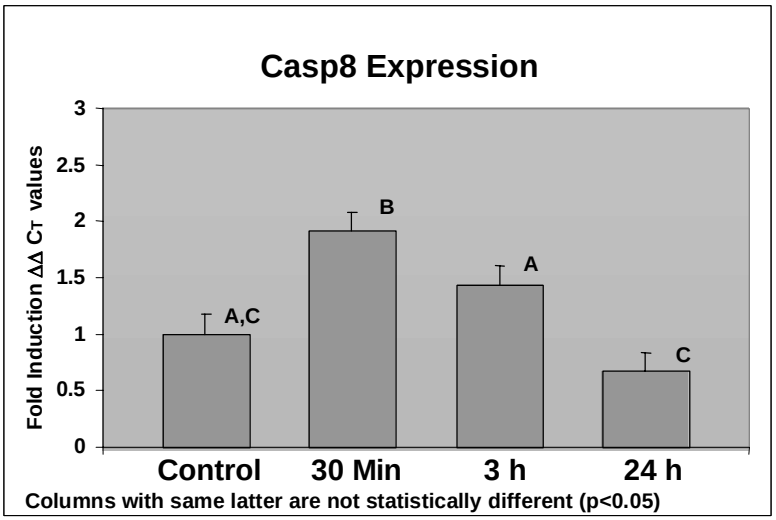


Figure 23. Relative expression of caspase 8 using β-actin as the calibrator gene. Caspase 8 showed an initial increase in expression 30 minutes after cloacal inoculation and gradually returned to control levels after 24 hours. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

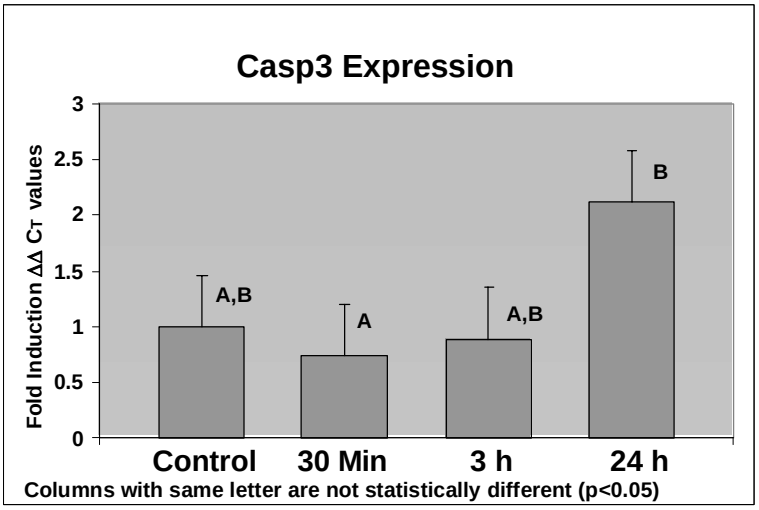


Figure 24: Relative expression of caspase 3 using β-actin as the calibrator gene. Caspase 3 expression levels are statistically the same at all time points sampled compared to the saline inoculated control, but the expression level after 24 hours is more than two-fold increased ($p<0.05$) compared to the expression after 30 minutes. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

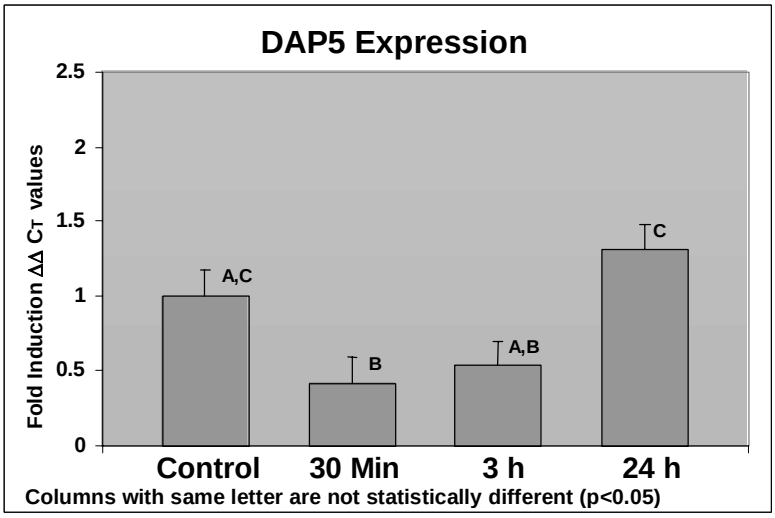


Figure 25: Relative expression of DAP5 using β-actin as the calibrator gene. DAP5 expression decreased initially to less than half the control level 30 minutes post inoculation ($p<0.05$) and gradually returned to control levels 24 hours post-challenge. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

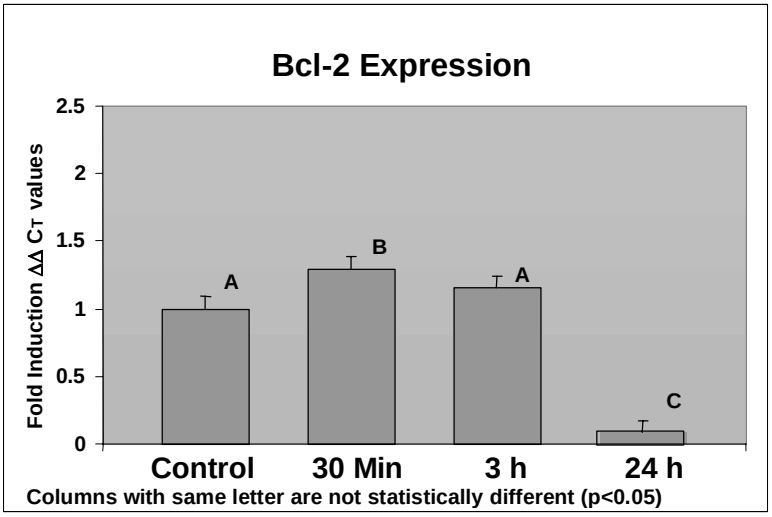


Figure 26. Relative expression of Bcl-2 using β-actin as the calibrator gene. The expression of B-Cell Lymphoma-2, an anti-apoptotic gene, is slightly up regulated initially at the 30 minute time period, but is dramatically decreased at 24 hours *versus* the saline inoculated control. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

Discussion

The chicken model plays an important role in the study of the humoral immune system, since birds have a distinct organ for the differentiation and maturation of B-cells. Chickens have the unique ability to sample their environment by way of cloacal drinking, by which material that gets trapped by the anal lips is retrogradely transported from the cloaca to the lumen of the bursa. Our study examined the molecular phenomena that take place in the bursa of Fabricius in the earliest stages of this type of environmental stimulation, in this case by inoculation of live *Salmonella*, a gram-negative bacterium that displays lipopolysaccharides (LPS) on its cell wall. The two most important findings of this study are (1) the eminent role of IL-12 during the early response phase and (2) the shift of the apoptotic balance towards increased apoptosis.

LPS is a classical example of pathogen-associated molecular pattern (PAMP) that upon interaction with a pattern recognition receptor (PRR), causes activation of the innate immune system as well as cellular stress responses. (Prohaszka and Fust, 2004). Recognition of LPS by TLR2/4 leads to an inflammatory response, characterized by the up-regulation of pro-inflammatory cytokines. In mammals, the main source of IL-12 seems to be the phagocytic, antigen-presenting cells (i.e. macrophages and dendritic cells). In infections caused by viruses, bacteria, and intracellular parasites, Il-12 is produced within a few hours of infection. It acts as a pro-inflammatory cytokine inducing interferon γ (IFNγ) production, which in turn enhances the phagocytic and bactericidal activity of phagocytic cells and their ability to release IL-12 and other pro-inflammatory cytokines (Balu and Kaiser, 2003).

In this study, IL-1β, IL-12β and IL-16 were investigated. Attempts to measure IFNγ were futile due to difficulties to develop a working set of real time PCR primers for IFNγ. While the increases of IL-1β and IL-16 were modest and delayed - their expression levels indeed transiently decreased prior to increasing with 50% or less (as compared to time 0) after 24 hours - a swift, dramatic and persistent up-regulation of the relative expression of IL-12β was the most noteworthy cytokine response (Figure 17). The bursal expression of IL-12β as measured in the RNA extracted from the complete organ - was increased five-fold within 30 minutes post-inoculation. Not only is this a surprisingly fast response, it also raises the question which cell type of the bursa of Fabricius was responsible for this fast and striking response. Since apparently only the medullary compartment of the bursa is influenced by environmentally derived antigens, the theoretical possibilities include the medullary B-cells, macrophages and dendritic cells (de Azevedo and Betti, 1993). In an attempt to identify bursal macrophages and/or adherent dendritic cells as the IL-12 source, the adherent bursal cells (devoid of B-cells and other non-adherent cells) were stimulated with LPS *in vitro*, but this did not seem to induce the expected up-regulation of IL-12 (Figure 18). In order to determine the approximate percentage of the B-cell population with in the bursal tissue, we previously utilized trypan blue staining to evaluate the number of live cells in a single cell suspension immediately following and 24 hours after cell collection. Our findings indicate that the B-cell population accounts for approximately 80% of the total bursal cell population, leaving the stromal cells to make-up around 20% of the cell numbers. This almost inevitably points to the B-cells of the medulla as the source of IL-12β

mRNA. According to Degen et al. (2004), IL-12 can indeed be induced in chicken DT40 pre-B-cells (*in vitro*) by stimulation with 10 μg/ml LPS. The same stimulus also induced IL-12 (p40) expression in HD-11 macrophages, but not in splenocyte cultures, CU91 T-cells or chicken embryonic fibroblasts. In mammals, production of IL-12 by B-cells is a matter of debate. Gurey et al. (Guery et al., 1997) reported that normal B-cells fail to secrete interleukin-12, but expression of IL-12β by B-cells has been documented in humans following CD40 stimulation (Airoldi et al., 2002) and in mice upon stimulation with CpG (Shirota et al., 2002).

To the best of our knowledge, this is the first physiological study reporting stimulation of IL-12 expression in the chicken *in vivo*. However, direct evidence about up-regulation of IL-12β expression of the bursal B-cells remains elusive, partly because culture of pure primary bursal B-cells is a major technical challenge. Previous studies have shown that within 6 hours, 80% of the chicken B-cells in cell culture die via apoptosis (Compton and Waldrip, 1998; Neiman et al., 1994). It may, however, be possible to further assess this issue by use of colorimetric (*i.e.* high resolution) *in situ* hybridization. We also need to solve the technical problems related to IFNγ mRNA measurement in order to test the hypothesis that IFNγ secreted upon IL-12 stimulation enhances the bactericidal functions of the medullary macrophages.

While the increases in IL-16 and IL-1β gene expression are in line with the current ideas about LPS stimulation of the innate immune system, they were very modest in comparison to the increase in IL-12β expression, and only occurred 24 hours post-challenge. Both IL-1β and IL-16 are important players in the inflammatory

response and are produced by a variety of immune cells, including B-cells (Delaleu and Bickel, 2004(Min, 2004 #38). IL-1β has been studied recently in chickens, but very little is known about its role in the bursa, where currently, only sequence information is known about IL-16 in the chicken (Delaleu and Bickel, 2004; Min and Lillehoj, 2004).

The other major observation that resulted from this study was a consistent shift of the balance between pro- and anti-apoptotic genes towards enhanced apoptosis. At the very least, these data contradict our initial hypothesis that stimulation of bursal lymphocytes with environmental antigen might increase the B-cell survival by reduction of the rate of apoptosis. Most strikingly, the average bursal expression of the anti-apoptotic gene Bcl-2 was reduced 12-fold within one day after challenge with *Salmonella*. At the same time, we observed an increase in the expression of genes that initiate (caspase 8) and execute (caspase 3) the apoptotic cascade, although these increases are at most 2-fold as compared to controls. Since these measurements reflect the average gene expression of the total bursa, the same question arises as to which might be the cell type(s) that are subject to apoptosis. Expression of Bcl-2 mRNA is low in the developing B-cells of the bursa of Fabricius (Lampisuo et al., 1998), whereas Bcl-x (a pro-apoptotic gene) is constantly detected in bursal cells of all ages, in line with the high susceptibility of bursal cells to apoptosis. Bcl-2 up-regulation has been reported in mammalian germinal centers where antigen-selected B-cells increase the expression of Bcl-2 and manage to escape apoptosis (Liu et al., 2004). In addition, lipopolysaccharide protects primary B lymphocytes from apoptosis by preventing mitochondrial dysfunction and bax translocation to mitochondria (Souvannavong et al., 2004). However, we did

not find any evidence in favor of this scenario in this study, although it cannot be excluded that it may take place at a later stage post-inoculation. Indeed, de Azevedo and Betti (1993) observed a great number of apoptotic nuclei from lymphoid cells in the cytoplasm of medullary macrophages 4 days after bursal inoculation with *Bordetella pertussis* (de Azevedo and Betti, 1993).

On the other hand, Winau et al.(Winau et al., 2004) recently proposed a host defense mechanism whereby infection with intracellular pathogens (including *Salmonella*) could lead to apoptosis of macrophages (Yrlid and Wick, 2000), producing apoptotic blebs carrying bacterial antigens to non-infected dendritic cells, ultimately leading to MHC-I mediated $CD8^+$ T-cell intervention.

The cellular response to a stressor introduced into the system leads to transient decreases in the synthesis and transcription of heat shock protein DNA (Prohaszka and Fust, 2004). In our study, HSC70 had an initial decrease at 30 minutes, before it increased significantly (i.e. 2-fold) at 24 hours post-challenge. Hsp90 and Hsc70 are both molecular chaperones that aide in protein folding (de la Rosa et al., 1998; Kang et al., 1999; Morales et al., 1998) Hsc70 helps to protect the cell from physical or environmental stress (de la Rosa et al., 1998; Morales et al., 1998). The increased expression suggests a potential protective mechanism to the stress of bacterial exposure.

In conclusion, upon cloacal inoculation with live bacteria, the shifts in the genes that were included in this study demonstrate the rapid initiation of an innate immune response (as shown by the increase in IL-12) followed by a shift of the apoptotic balance

in favor of enhanced programmed cell death. Exactly which cell type is responsible for which of the observed effects is a topic of future investigation.

CHAPTER VI

DOWN-REGULATION OF SERINE PROTEASE INHIBITORS IN THE STROMAL CELLS OF THE BURSA OF FABRICIUS : IS UP-REGULATION OF SERINE PROTEASES INVOLVED IN THE EARLY PHASE OF THE IMMUNE RESPONSE?

Introduction

The bursal secretory dendritic cells (BSDC) are believed to be important non-lymphoid players in the maturation of B-cells in the bursa of Fabricius (Glick, 1995). The monoclonal antibody against vimentin, an intracellular protein, labels the BSDC, with an intense staining in the cortico-medullary region of the follicle (Olah et al., 1992a; Olah et al., 1992b). Previous studies have shown that the vimentin-positive BSDC, the ovoinhibitor-positive and the TrkB-labeled cells share the same distribution pattern, *i.e.* they are most concentrated in the cortico-medullary border region (Ciriaco et al., 1997; Moore et al., 2004). In chapter VII, the secretion of inter-α-trypsin inhibitor H3, another serine protease inhibitor, and serine protease inhibitor clade A (SPICA) were identified by the stimulation of the *in vitro* adherent cell population with LPS from *Salmonella*.

Serine proteases play a major role in the processing and presentation of antigens, which may be a process the BSDC, as well as macrophages, are involved in (Steinhoff et al., 2005). For instance, PMSF, a serine protease inhibitor inhibits presentation of viral

antigens by macrophages to B-cells (Rizvi et al., 1991). Cathepsin G is a serine protease that is a major functional constituent of the proteolytic machinery of B-lymphocytes (Burster et al., 2004). Serine protease inhibitors play a role in competitively inhibiting the enzymatic activity of these enzymes (Ye and Goldsmith, 2001). Thus, we hypothesize that serine protease inhibitors will be affected, likely down-regulated, during the innate immune response provoked by cloacal inoculation with *Salmonella* or stimulation with LPS *in vitro.*

In all living organisms there is a delicate physiological balance (homeostasis) between anabolic and catabolic processes (production and destruction) at all times. This is also true for the balance between protease inhibitors (inhibiting proteins) and the proteolytic enzymes they inhibit (Steinhoff et al., 2005). Serine protease inhibitors have a broad scope of functions and are the major protease inhibitors found in plasma of vertebrates, where they regulate diverse processes, including blood coagulation, fibrinolysis and inflammation (Patston et al., 1994; Potempa et al., 1994; Vassalli et al., 1991). Serpins have also been described in endocrine tissues and in the brain. Examples include kallistatin, expressed in the adrenal gland (Ecke et al., 1992) and Endopins I and II, expressed in the adrenal medulla, the pancreas and the pituitary (Hwang et al., 1994; Hwang et al., 1995; Hwang et al., 1999b). One serine protease inhibitor, ovoinhibitor, has a cellular staining pattern similar to that of the BSDC; the cognate enzyme of the inhibitor has not been identified, however, and its true function in the immune response is unknown at this time. Previous research has shown the localization of ovoinhibitor, a Kazal type serine protease inhibitor, in both endocrine and immune tissues, *i.e.* the

pituitary (Oubre et al., 2003) and the bursa of Fabricius (Moore et al., 2004), respectively.

The function of another serine protease inhibitor, inter-α-trypsin inhibitor H3 (IATI3), a molecule we came across during a proteomic study of molecules secreted upon LPS stimulation of the bursal adherent cells, is even less understood in the context of the chicken bursa of Fabricius model. Unlike the situation for ovoinhibitor, the cellular distribution of IATI3 in the cells of the bursa has not been investigated. Also, the cognate enzyme has not been identified in the context of the bursa of Fabricius, but in its capacity as a serine protease inhibitor, it may be affected during the innate immune response. Inter-α-inhibitors (IαI) are generally referred to as protease inhibitors of the blood plasma (Salier et al., 1996). IαI have recently been described in acute inflammation reactions, leading to both up- and down-regulation of acute-phase proteins. The IαI is a selective combination of three heavy chains H1, H2, H3 and bukinin, a light chain, with H3 having an up-regulatory influence on acute phase protein production and H1 and H2 down-regulating acute phase proteins (Salier et al., 1996; Zhuo et al., 2004). IαI are found in the plasma, with most circulating forms produced by the liver.

TrkB, a neurotrophin receptor, has a potential link with the BSDC, since cells that are stained with the antibody against TrkB receptor are located in the medullary area of the bursal follicle and are morphologically similar to the BSDC population (Ciriaco et al., 1997). TrkB is one of several neurotrophin receptors in the Trk family of proteins. They are composed of tyrosine kinase proteins that have been found in lymphoid organs of mammalian species (Laurenzi et al., 1994; Lomen-Hoerth and Shooter, 1995). Their

localization in the bursa provides a potential neuroendocine-immune function of the cells that express these receptors. We were interested in the expression levels of the TrkB receptor during LPS stimulation.

Our current research was directed towards the evaluation of serine protease inhibitor gene expression of the adherent cell population following LPS stimulation. Previous studies have shown that within 6 hours, 80% of the chicken B-cells in cell culture die via apoptosis (Compton and Waldrip, 1998; Neiman et al., 1994). Our study focuses on evaluating a B-cell rich model using *in vivo* cloacal inoculation of chicks and a B-cell depleted, adherent cell system that is selected by overnight attachment to cell culture flasks. TrkB was included in this study because of its link to the BSDC population by immunohistochemical detection of positive cells (Ciriaco et al., 1997). Regulation of this neurotrophin receptor message could mean that the cells become more or less sensitive to exogenous neurotrophin, but the link with antigen presentation or immune response is not at all clear. It is included in this study in order to evaluate both molecules that have been linked to the BSDC population by cellular localization.

Materials and methods

Immunohistochemistry

Paraffin embedded, 4% (w/v) paraformaldehyde fixed bursal tissue sections of 1-13 week old birds were made using a rotary microtome (RMC MT 980). Tissues were dewaxed and rehydrated by two incubations in xylene for 5 minutes followed by each of the following for 5 minutes each, 95% ethanol, 70% ethanol, 50% ethanol, and dH_2O.

Enzymatic staining procedures began by rinsing the tissue sections with Tris-buffered saline containing 0.1% Triton-X (TBST) followed by incubation with normal goat serum (Sigma, St. Louis, MO; 1:10 dilution in TBST) for 1 hour. The tissue sections were then incubated overnight with the anti-ovoinhibitor antibody (Moore, 2004, ascites fluid at a 1:9000 dilution). The slides were rinsed the next morning using TBST and incubated with horseradish peroxidase-conjugated goat-anti mouse Ig (Jackson, West Grove, PA) at a dilution of 1:500 in TBST for 1 hour. The sections were rinsed with PBS four times 5 minutes followed by a 5 minute incubation in a 50 mM Tris-HCl buffer (pH 7.4, no Triton). DAB (Sigma) at 25 mg and 75 μl of 30% H_2O_2 were added to 200 ml of Tris-HCl buffer for enzymatic color detection. The reaction was stopped by transferring the sections to dH_2O. Coverslips were applied using cryoseal XYL (Stephens Scientific, Kalamazoo, MI) and viewed using bright field light microscopy (Olympus BX50 light microscope; Leeds Instruments, Inc., Irving, TX) and photographed with a Spot 110 digital camera (Diagnostic Instruments, Inc., St. Sterling Heights, MI).

In vivo bursal inoculation and tissue sampling

Four-day old white leghorn male chicks (Hy-Line International, Bryan, TX) were challenged intra-cloacally with 0.5 ml of a 10^5 cfu/ml culture of *Salmonella enterica* serovar Typhimurium, (Presque Isle Cultures, Presque Isle, PA). Four groups of three birds were used, three of which were cloacally inoculated with bacteria and one group was given sterile saline as the unchallenged control. Bursal tissue was collected and stored in RNAlater (Ambion, Austin, TX) at 30 minutes, 3 hours, and 24 hours post-challenge; control samples were collected at 24 hours. Three individual bursae from each time point were homogenized and total RNA was extracted using the Qiagen RNeasy Kit (Qiagen, Valencia, CA). Following RNA extraction, reverse transcription was performed with the RETROscript cDNA kit using oligo dT (Ambion Austin, TX).

In vitro cell collection and stimulation

In order to discriminate between B-cells and adherent cells as the potential source of the gene shifts observed *in vivo,* adherent cells were enriched by overnight incubation of a bursal single cell suspension. The adherent cell population was considered free of B-cells since bursal B-cells are non-adherent and quickly undergo apoptosis upon disruption of the bursal intercellular contacts (Neiman et al., 1994). The bursa of Fabricius was removed from day old white leghorn chicks (Hy-line Bryan, TX). Tissue was digested by three two-hour incubations with 2μg/ml collagenase (Invitrogen, Carlsbad, CA) at 37^{o}C while shaking. Cells were collected and placed in T75 vented flasks (Becton Dickson, Menlo Park, CA) overnight. The next day non-adherent cells

(mostly dead B-cells) were removed and the flasks were rinsed with sterile PBS (Sigma, St. Louis, MO). Samples were stimulated with growth media containing 5 μg/ml of LPS from *Salmonella enterica* serovar Typhimurium, and a control sample was used that contained growth medium only in a separate flask. After 3 hours the cells were collected and RNA was extracted using the Trizol method by adding 2 ml of Trizol to each flask on ice. After a five minute incubation, the Trizol and cells were collected and frozen at 20^{o}C. Chloroform was added and the upper aqueous phase was collected. Isopropanol was added to create an RNA pellet, which was washed with ethanol and then air-dried. Once dry, 30 μl of nuclease-free H_20 was added and the sample was stored at -20^{o}C until reverse transcription.

Quantitative PCR

Serine Proteinase Inhibitor, clade A (SPICA1), TrkB Neurotrophin Receptor (TrkBR), and inter alpha-trypsin inhibitor, heavy chain 3 (IATI3) primers were designed using the published ESTs (genome.ucsc.edu), assembled with CAP3 software (Huang and Madan, 1999) and designed using Primer express software (Applied Biosystems; Foster City, CA), specific primer sequences are listed in Table 4. Primers for the amplification of ovoinhibitor (OI) cDNA were designed using the published sequence gene bank sequences. All primers were ordered from Integrated DNA Technologies, Inc.(Coralville, IA) Reverse transcription was performed on the RNA samples from both the *in vivo* and *in vitro* experiment with the RETROscript cDNA kit using oligo dT (Ambion Austin, TX). A sample concentration of 1 μg cDNA per tissue sample and

Sybr Green PCR Master Mix (Applied BioSystems, Foster City, CA) was used for the PCR reaction using gene-specific primers. The amount of template was consistent in each sample and chicken β-actin primers were used for normalization purposes (Thellin et al., 1999). All *in vivo* samples were run in duplicate and the *in vitro* samples were run in triplicate. The QPCR specifics include an initial denaturation temperature of 95^{o}C for 10 minutes followed by forty cycles of a 15 second hold at 95^{o}C then a 1 minute hold at 60^{o}C. Ct values of each gene and treatment were used to determine fold induction of the gene according to the ΔΔCt method (Lehmann and Kreipe, 2001; Livak and Schmittgen, 2001). Analysis of variance was performed and significant differences between means were determined by Least Squares Means method using SAS ($p<0.05$) to determine the relative quantification of all target genes.

Table 4: Primer sequences designed for QPCR of serine protease inhibitors and neurotrophin receptor, TrkB. SPICA1, Serine protease inhibitor clade A; TrkBR, TrkB receptor; IATI3, Inter-alpha- trypsin inhibitor 3; OI, ovoinhibitor primer sequences.

Gene Target	**Upstream 5'-3'**	**Downstream 5'-3'**
SPICA1	AACCAGGTAGAAGATGCTCTGCTA	GTCCGTCACACCCATTTTCAA
TrkBR	CGACCAGCTGAGCTGACACA	CGACCAGCTGAGCTGACACA
IATI3	GAACCAGGTAGAAGATGCTCTGCTA	GTCCGTCACACCCATTTTCAA
OI	AGGATGGCAGGACTTTGG	GTGCAGATGGGAGAGAGACT

Primer specificity verification

In order to verify the length of the quantitative PCR products, the amplicons were verified separated on a 2% agarose E-gel, stained with ethidium bromide and analyzed under UV light.(Invitrogen, Carlsbad, CA).

Results

Immunohistochemistry

The immunohistochemical staining procedure produced with the anti-ovoinhibitor monoclonal antibody revealed cytoplasmic staining of cells in both the cortex and medulla of the bursa of Fabricius, with a large cell concentration in the cortico-medullary border (Figure 27). The pictures seem to indicate two different types of staining. Either the we see the typical crisp "donut-like" labeling that is typical for a molecule that is stored in large quantity in the cytoplasm; this is especially clear for the cells on the cortico-medullary border (Figure 27B), and also in the scattered positive cells in the medulla. The staining pattern in the cortex is much weaker and less clear cut, and looks more like a smear covering all cells of the cortex.

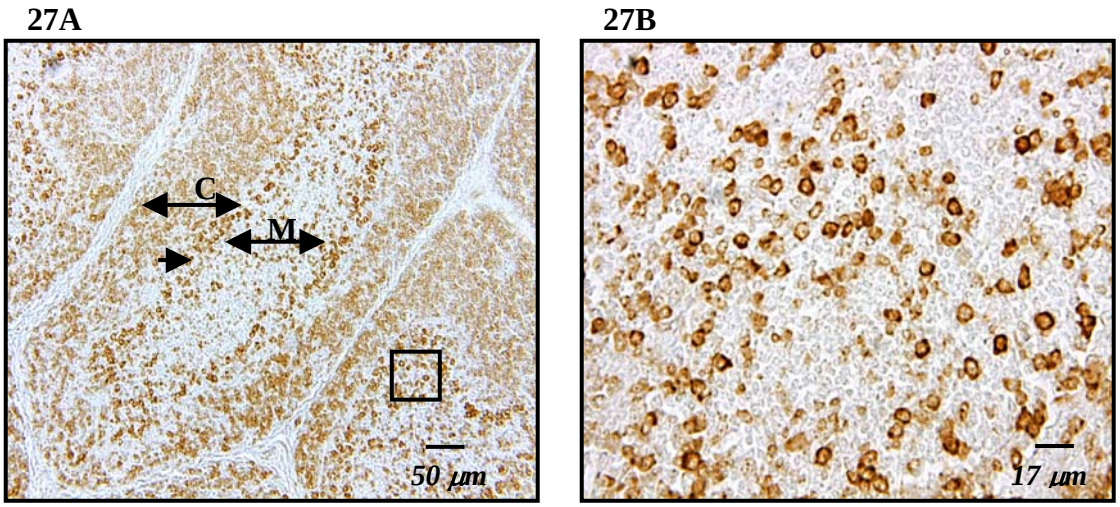


Figure 27: Immunohistochemical staining of the bursa of Fabricius using a monoclonal antibody against the ovoinhibitor protein.
A. An enzymatic staining of a bursal follicle, the cortex (C) and medulla (M) are sporadically stained, with intense staining in the cortico-medullary border, labeled with an arrow. (Bar equals 50 μm). The boxed area is shown at higher magnification in 1B.
B. An enzymatic staining of the cortico-medullary border region of a bursal follicle. (Bar equals 17 μm).

Primer verification

The agarose gel displays amplicons at the expected range for the OI and IATI3 primer pairs used (Figure 28). The OI amplicon is expected to be 850 base pairs in length based on the published sequence of ovoinhibitor (Scott et al., 1987), while the IATI3 and SPICA amplicons should be between 100 - 250 base pairs in length. A much larger band was seen in the SPICA amplicon, after sequencing, it was determined that this was not the correct amplicon and the results of SPICA primers were excluded from further analysis.

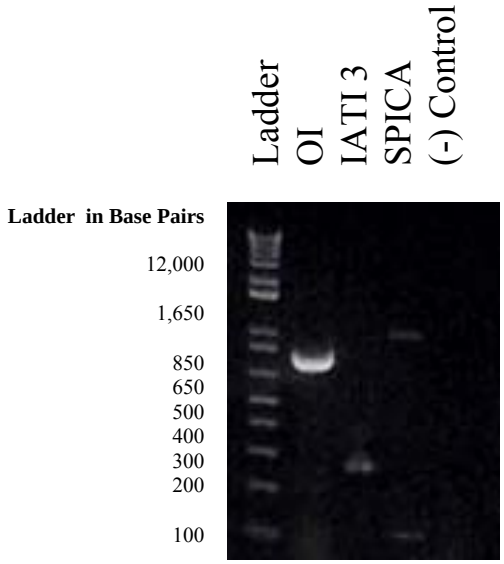
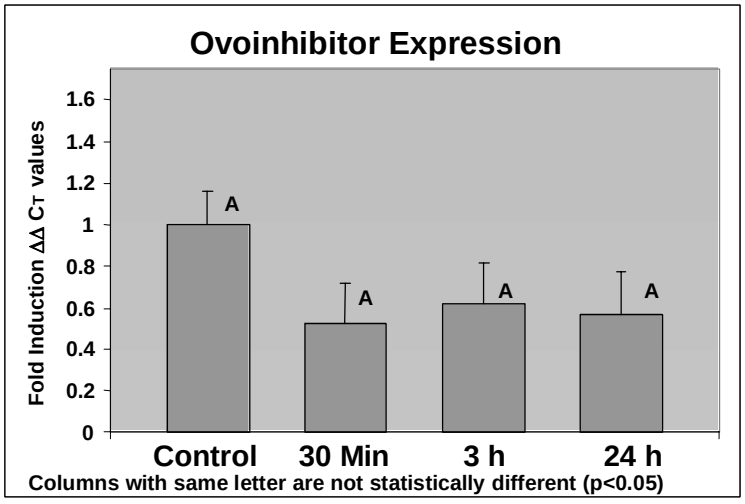


Figure 28: 2% agarose gel stained with ethidium bromide to visualize PCR amplicon size. OI band is in the expected range of approximately 850 base pairs and IATI3 is approximately 250 base pairs in length. The SPICA amplicon has a band in the expected range, but contains a much larger amplicon as well. TrkB amplicon verification is shown in chapter V.

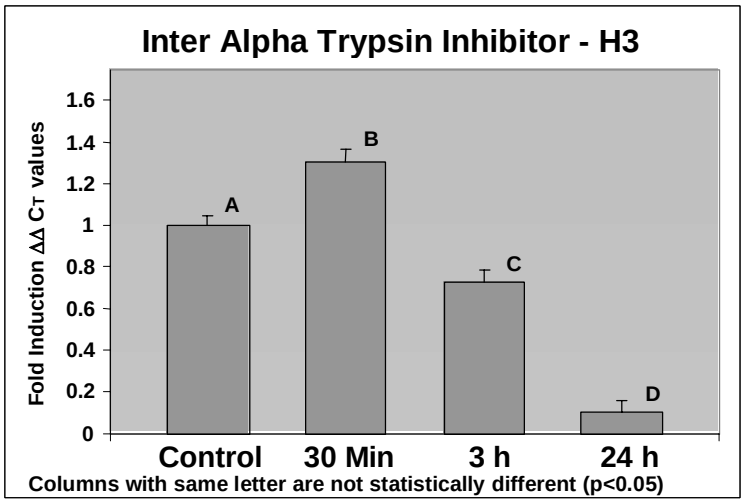
QPCR of bursal RNA samples obtained after *in vivo* cloacal inoculation

The gene expression levels of ovoinhibitor show a decreasing numerical trend, but the expression is not significantly different from that of the controls at any time point due to the large variation between the different animals (Figure 29). By contrast, the expression of the inter alpha trypsin inhibitor – H3 gene (IATI3) showed a prompt, but moderate increase in expression level at 30 minutes followed by a gradual but steady decrease to as little as approximately 10% of control levels 24 hours post-inoculation (Figure 30).



29.

Figure 29: Relative expression of ovoinhibitor using β-actin as calibrator gene, bursal mRNA samples were obtained from birds that had been cloacally inoculated with *Salmonella enterica* serovar Typhimurium. While there was a decreasing numerical trend, no significant difference was seen between controls and test samples at any time points. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.



30.

Figure 30: Relative expression of Inter alpha trypsin inhibitor (H3 chain) using β-actin as calibrator gene, bursal mRNA samples were obtained from birds that had been cloacally inoculated with *Salmonella enterica* serovar Typhimurium. IATI3 expression data display a moderate transient increase in expression (at 30 minutes) followed by a rapid and steady decrease down to 10 % of control levels at 24 hours post-inoculation. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

QPCR of bursal RNA samples obtained after *in vitro* challenge of the bursal adherent cells with LPS

In contrast to what is seen in the *in vivo* samples, the relative gene expression levels as measured by real-time PCR in he samples obtained from *in vitro* LPS stimulation show decidedly decreased expression for both protease inhibitor genes. The ovoinhibitor gene expression level was decreased spectacularly, i.e.approximately 65-fold lower compared to control samples (Figure 31). The inter alpha trypsin inhibitor – H3 levels were found to be more than 2-fold decreased from the control samples (Figure 32).

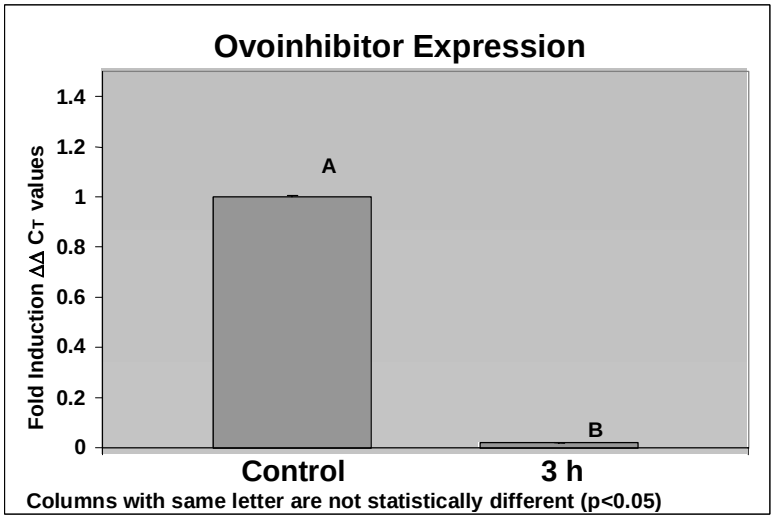


Figure 31: Relative expression of ovoinhibitor three hours after *in vitro* stimulation of the adherent cell population with 5 μg/ml LPS, β-actin was used as calibrator gene. The expression level of ovoinhibitor were dramatically decreased *versus* the un-stimulated control samples. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

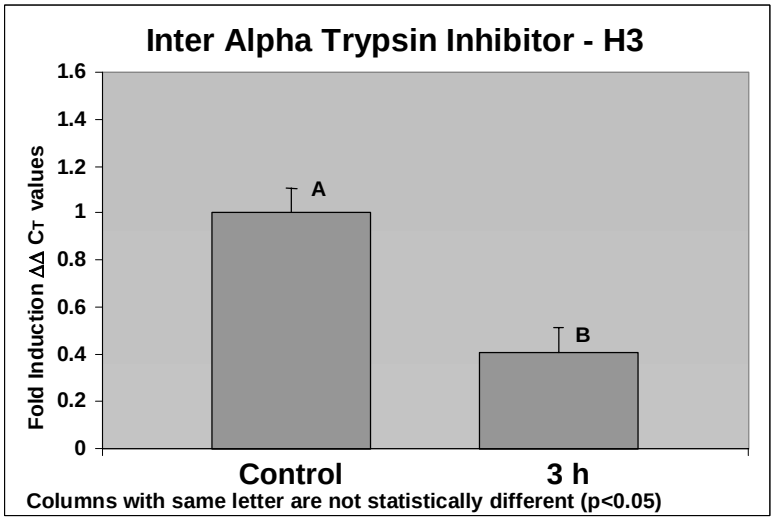
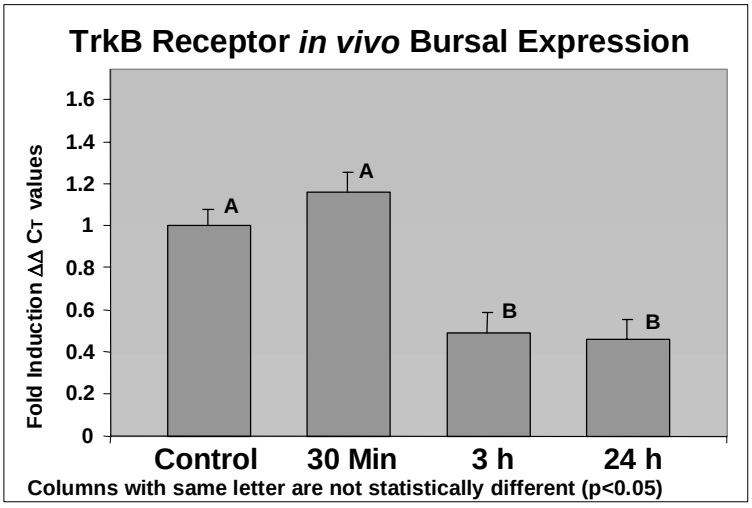


Figure 32: Relative expression of inter alpha trypsin inhibitor – H3 three hours after *in vitro* stimulation of the adherent cell population with 5 μg/ml LPS, β-actin was used as calibrator gene. The relative expression levels of IATI3, were decreased significantly *versus* control levels post-stimulation. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

TrkB neurotrophin receptor

The neurotrophin receptor TrkB expression levels in the *in vivo* samples decreased 2-fold by 3 hours post inoculation and stayed under control levels through 24 hours after bursal inoculation with Salmonella *in vivo* (Figure 33A*).* The gene expression levels of TrkB in the samples obtained from the adherent bursal cells after *in vitro* stimulation with LPS (5 μg/ml), displayed an approximate 3.5-fold expression level decrease at three hours post stimulation with LPS *versus* the control levels (Figure 33B).

A.



B.

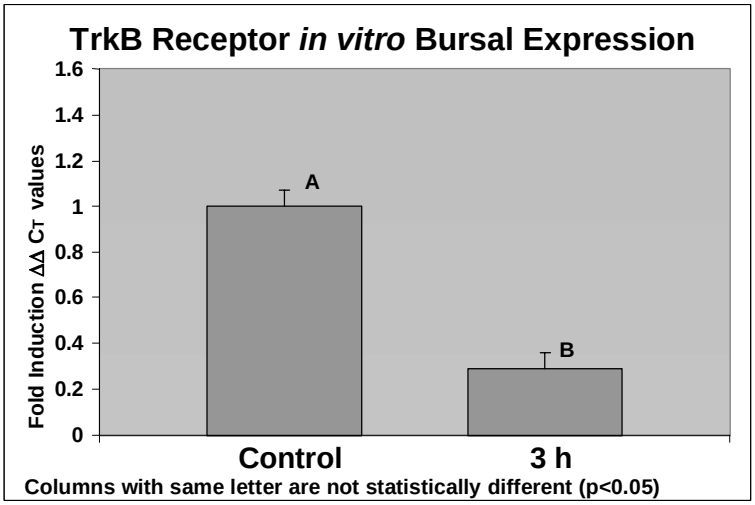


Figure 33: Neurotrophin receptor, TrkB, gene expression levels *versus* control of both *in vivo* and *in vitro* stimulation. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.
A. TrkB Receptor Expression decreases significantly by 3 hours after inoculation and is sustained through 24 hours.
B. The gene expression levels of TrkB receptor, neurotrophin receptor, were drastically reduced at three hours post stimulation with LPS versus the control levels. Error bars represent standard error within the treatments.

Discussion

In this study, we evaluated the expression of serine protease inhibitors and a neurotrophin receptor *in vivo* and *in vitro*. The *in vivo* cell populations were stimulated intra-cloacally with live *Salmonella*, while the *in vitro* populations were enriched by adherence to plastic, followed by stimulation with LPS from *Salmonella*. Both sample populations were collected and QPCR was performed to evaluate specific protease and neurotrophin receptor gene expression. The gene expressions of OI and IATI3 of the *in vitro* derived, adherent cell population was affected greatly by stimulation with LPS, while the OI expression levels of the *in vivo* samples were not significantly different from the control levels. Since a majority of the *in vivo* cell population are B-cells, which are absent from our *in vitro* system, it is possible that they mask the gene shifts *in vivo* (Compton and Waldrip, 1998; Neiman et al., 1994). Unlike the protease inhibitors, the TrkB neurotrophin receptor displayed decreases in both the *in vivo* system (at 3 and 24 hours post challenge) and in the *in vitro* model (after a 3-hour exposure to LPS).

Protease inhibitors and the enzymes they regulate are in a state of balance. If there is a decrease in protease inhibitor production, an increased enzymatic protease activity is likely to occur, while an increase in protease inhibitor production indicates a potential decrease in protease activity. In this study, the introduction of LPS to the adherent population yields a significant decrease in protease inhibitor gene expression, in one case more than 65-fold. As a result, the specific proteases in the bursa may play a potential role in cellular activation following recognition of a foreign substance, such as

in this case LPS. These proteases may play a role in destruction of the pathogen and/or antigen presentation. If they affect antigen presentation, a potential influx of T-cells into the bursal tissue is needed, giving the bursa a secondary immune function, along with the primary immune function.(Yasuda et al., 1998) This is the first study to identify the production of serine protease inhibitors by the adherent cell population in the bursa of Fabricius. Further characterization of the protease inhibitors and the proteolytic enzymes they inhibit needs to be done to completely understand their link with immune challenge.

TrkB receptor expression was also studied to evaluate the gene expression of the other molecule that have been linked to the BSDC population in the bursa of Fabricius, following live *Salmonella* exposure *in vivo* and LPS stimulation *in vitro*. The TrkB receptor belongs to the Trk family of neurotrophin receptors that are composed of tyrosin kinase proteins that have been found in lymphoid organs of mammalian species.(Laurenzi et al., 1994; Lomen-Hoerth and Shooter, 1995) The expression of the neurotrophin receptor is directly linked to the ability to act in response to the presence of the corresponding neurotrophin (Ip and Yancopoulos, 1994; Ip and Yancopoulos, 1996). The neurotrophin receptors have been recently identified by immunohistochemistry in the chicken bursa of Fabricius, suggesting a newly identified area of neuroendocrine influence in the bursa (Ciriaco et al., 1997). TrkB receptors bind and are activated by brain-derived neurotrophic factor (BDNF) and neurotrophin 4/5 (NT4/5), which are involved in nerve growth and survival processes (Guiton et al., 1994; Ip and Yancopoulos, 1994; Ip and Yancopoulos, 1996). Previous studies have identified the

release of nerve growth factors by mast cells and the localization of TrkB in the ovarian follicle as well as the developing embryo (Jensen and Johnson, 2001; Skaper et al., 2001). In our study, TrkB displayed a marked decrease in expression around two fold below control levels during *in vivo* stimulation indicating a decrease in potential signal transfer 3 hours post-stimulation, continuing through 24 hours. TrkB displayed a 3 fold decrease in gene expression ($p<0.05$) at 3 hours post-stimulation in the *in vitro* LPS stimulated samples compared to the control. A study by Ciriaco et al. localizes the TrkB receptor in the bursa of Fabricius using immune staining of the tissue, specifically the bursal secretory dendritic cells were stained, elucidating a potential neuroendocine-immune function of these cells (Ciriaco et al., 1997). Our findings confirm the molecular presence of the TrkB receptor in the bursa, although the exact function is still to be determined. This study is the first time a neurotrophin receptor has been identified using molecular methods in the chicken bursa of Fabricius.

In summary, we find decreased expression of both serine protease inhibitors and neurotrophin receptors following the stimulation of the adherent cell population from the bursa of Fabricius. This decrease may result in the increased activity of the corresponding enzyme(s) the protease inhibitors inhibit. Previous studies have identified ovoinhibitor and TrkB to be present in the BSDC population of the bursa, mainly in the cortico-medullary border region of the follicle (Ciriaco et al., 1997; Moore et al., 2004). The evaluations of the expression of these genes, further characterizes the BSDC cell population. Further analysis is needed to understand this relationship and how it affects the immune response of the bursa.

Future studies should include the culturing of HD-11 cells, an avian macrophage cell line, by stimulating with LPS to evaluate the specific response of the macrophages compared to the response of the total adherent cell population consisting of macrophages, BSDC, and other potential adherent cells. This would allow the responses of the macrophage cells to be evaluated in conjunction with the adherent cell population for identification of potential shared and separate responses to immune stimulation.

CHAPTER VII

PROTEIN SECRETORY PATTERN AND GENE EXPRESSION OF THE ADHERENT CELL POPULATION FROM THE CHICKEN BURSA OF FABRICIUS IN RESPONSE TO STIMULATION WITH LPS FROM *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

Introduction

The Bursa of Fabricius, the site of B lymphocyte maturation and development, is unique to the avian species (Glick and Olah, 1987a). The events involved in the interaction between the bursal microenvironment and the development and maturation of the B-cells are not well understood. It is known that stromal, or non-lymphoid cells are involved in this maturation process, but exactly which role they play is still unclear. What is well-known by now is the fact that bursal B-cells undergo apoptosis within a matter of hours as soon as they are removed from their micro-environment (Compton and Waldrip, 1998). This may be due to the loss of inter-cellular contacts, but also by lack of humoral factors produced by the stroma. One of such humoral factor was recently identified as B-cell activating factor (BAFF) (Koskela et al., 2004; Mecklenbrauker et al., 2004; Schneider et al., 2004). BAFF (BlyS, TALL-1). It is a tumor necrosis factor family member, is mainly produced by myeloid and dendritic cells and promotes B-cell differentiation and survival in a paracrine fashion. It was shown recently that BAFF is up-regulated at the bursal stage of the avian B-cell development

and that it also functions to promote bursal B-cell survival in an autocrine manner (Koskela et al., 2004).

In addition to various other cell types, the Bursal Secretory Dendritic-like Cell (BSDC) is a part of this stromal environment and is postulated to influence humoral immune development, although the mechanism of this interaction has never been demonstrated (Glick, 1994). BSDCs are identified using monoclonal antibodies against vimentin, an intermediate filament, and can be enriched by adherence to plastic (Glick and Olah, 1987a; Jeurissen et al., 1994b). The goal of the current study was to identify proteins released and/or synthesized by the adherent population of the Bursa in response to LPS exposure *in vitro*.

In order to evaluate the effects of *Salmonella enterica* serovar Typhimurium-derived LPS, we utilized the new model of enriching the BSDC by adherence to plastic. The protein production of *in vitro* enriched bursal cell samples was evaluated at 30 minutes, 3 hours, and 24 hours post-stimulation and compared to growth media only inoculated controls. Cell supernatants were collected and run on SDS-polyacrylamide gels under reducing conditions. Differentially expressed bands were excised and submitted to the Lab of Neuroplasticity and Neuroproteomics (K.U.Leuven, Belgium) for mass spectrometric analysis. Using the protein sequencing data, specific genes were further analyzed to determine if differential gene expression was up-regulated at three hours post-stimulation with LPS. The genes previously used in the *in vivo* experiments, IL-12β, IL-16, Hsp90, Hsc70, caspase 8, caspase 3, DAP5, and Bcl-2, were also evaluated by quantitative polymerase chain reaction (QPCR).

Materials and methods

Cell culture and adherent cell enrichment

Adherent cells were enriched by overnight incubation of a bursal single cell suspension. The adherent cell population was considered free of B-cells since bursal B-cells are non-adherent and quickly undergo apoptosis upon disruption of the bursal intercellular contacts (Compton and Waldrip, 1998; Neiman et al., 1994). The bursa of Fabricius was removed from day-old white leghorn chicks (Hy-line Bryan, TX). Tissue was digested by three, two-hour incubations with 2μg/ml collagenase (Invitrogen, Carlsbad, CA) in RPMI-1640 (Sigma, St. Louis, MO) at 37^{o}C while shaking. Cells were collected and placed in T75 vented flasks (Becton Dickson, Menlo Park, CA) overnight. The next day non-adherent cells (mostly dead B-cells) were removed and the flasks were rinsed with sterile PBS (Sigma, St. Louis, MO). Serum free RPMI-1640 (Sigma, St. Louis, MO) containing 5 μg/ml of LPS from *Salmonella enterica* serovar Typhimurium was added to separate flasks for 30 minutes, 3 hours or 24 hours; growth medium only was used as the control.

At the appropriate time the supernatant was collected and used for proteomic analysis and the cells were used for quantitative PCR. Protein concentrations were equilibrated between treatment groups.

Polyacrylamide gel electrophoresis

Samples (volumes normalized to contain identical amounts of total protein) were run on SDS polyacrylamide gels made with appropriate volume of a 30% acrylamide solution, 25% 1.5 M Tris pH 8.8, 10% APS, 10% SDS and 5 µl of TEMED for the running gel and 13% of a 30% acrylamide solution, 25% 1.0 M Tris pH 6.8, 10% APS, 10% SDS and 5 µl of TEMED (all reagents were obtained from Sigma, St. Louis, MO) under reducing conditions. Two gel percentages were made, 5% and 7.5%, 1 mm in thickness with dimension of 16x20 cm and run on a Protean II vertical gel electrophoresis system (BioRad, Hercules, CA). BioRad's Kaleidoscope standards were used as approximate molecular weight makers (Hercules, CA). The bands were removed from the gel, placed in dH_2O with 0.1% Formic Acid (Sigma, St. Louis, MO), and shipped for protein analysis.

Protein digestion, peptide purification and sequencing

The samples were analyzed in the Lab of Neuroplasticity and Neuroproteomics at the University of Leuven, Belgium by Dr. Stefan Clerens. Upon receipt, samples were rinsed in milli-Q water followed by rinses in a 50% Acetonitrile (ACN). The samples were desiccated by SpeedVac vacuum centrifugation and digested overnight at 37^oC in a buffer containing 100 ng of modified porcine trypsin, 12.5 mM NH_4HCO_3, and 5% ACN. After digestion the samples were rinsed in 5% ACN followed by 30 minute sonication to remove the protein from the gel fragment. Samples underwent desalting and concentration using ZipTip C18 reverse-phase chromatography (Millipore

Corporation, Bedford, MA). Protein analysis was performed by Q-TOF mass spectrometry (MS) (nano-ESI Q-TOF; Micromass Waters, Milford Massachusetts), and the resulting sequences were identified by BLAST search. Search criteria required a minimum of three sequence matching peptide fragments for identification.

Primer design and sequence for quantitative PCR

The complement component C3 (C3), complement component Factor B precursor (Factor B), alpha-2-HS-Glycoprotein (A2HSP), and interleukin 12 β (IL-12β) primers were designed using the published ESTs (genome.ucsc.edu), assembled with CAP3 software (Huang and Madan, 1999) and designed using Primer express software (Applied Biosystems; Foster City, CA). Primers for the amplification of interleukin 16 (IL-16), heat shock protein 90 (Hsp90), Heat Shock Cognate 70 (Hsc70), Death Associated Protein-like 5 (DAP5), Caspase 3 (Casp 3), Caspase 8 (Casp 8), and B-cell Lymphoma (Bcl-2) cDNA amplifications were designed using the published sequence gene bank sequences. Actin was used as the calibrator gene; every gene's expression level was expressed relative to the expression of actin (Thellin et al., 1999). All primers were ordered from Integrated DNA Technologies, Inc. (Coralville, IA). Primer sequences are listed in Table 5.

Table 5: Primer sequences used for quantitative PCR of adherent cell population. Complement component C3(C3), complement component Factor B Precursor (Factor B), Interleukin 16 (IL-16), Interleukin 12β (IL-12β), Caspase 3 (Casp 3), Caspase 8 (Casp 8), Death Associated-Like Protein 5 (DAP5), Heat Shock Protein 90 (Hsp90), Heat Shock Cognate 70 (Hsc70), Alpha-2-HS-Glycoprotein (A2HSP), and β-Actin Primer – calibrator gene.

	Forward Primer	Reverse Primer
C3	AAGGACAATCCCATCACAGTCAA	GGTTCTCATCAGTGCCCATCTT
Factor B	TGCTGGAGAAGCTCAACTTCAGA	TGTTGGACCAGCAGCGAATAG
IL-16	CTGCCACCCAGTCTTGTGAGA	ATGACACCTGGTTACTGATGGAATAG
IL-12β	TAAAGTAGACTCCAATGGGCAAATG	TAAAGTAGACTCCAATGGGCAAATG
Casp 3	GATGCTGCAAGTGTCAGA	ATCGCCATGGCTTAGCA
Casp 8	AGACTTCCTCTTGGGCATGACTA	TTGGCACAGTGACTGTATGTACCA
DAP5	ACAAGGACAGTCGAAGGATATGC	GCAGGTCTCAGGCTTATCTCATC
Bcl-2	TGAGCAGAGGTCACGTA	CACACTGTGGAACAGCA
Hsp90	GGCAGTTTGGTGTGGGTTTCT	GCTCGTCATCGTTGTGCTTG
Hsc70	TCATCAAGCACACAAGCCAGTA	AGCGAGCTCTGGTGATCGAT
A2HSP	TGTCTACCTCATGCTCCTTGAGATC	GATCCTCAGGAAGCAGTTGACAA
β-Actin (cal.)	CTGATGGTCAGGTCATCACCATT	TACCCAAGAAAGATGGCTGGAA

Quantitative PCR

The adherent cell population from the overnight *in vitro* enrichment was collected. RNA was extracted using the Trizol method by adding 2 ml of trizol to each flask on ice. After one five minute incubation, the Trizol and cells were collected and frozen at -20^{o}C. Chloroform was added and the upper aqueous phase was collected. Isopropanol was added to create an RNA pellet that was washed with ethanol then air dried. Reverse transcription was performed on the RNA samples with the RETROscript cDNA kit using oligo dT (Ambion Austin, TX). A sample concentration of 1 μg cDNA per tissue sample and Sybr Green PCR Master Mix (Applied BioSystems, Foster City, CA) was used for the PCR reaction using gene-specific primers (Table 1). As necessary

controls, the amount of template was consistent in each sample and chicken β-actin primers were used for normalization purposes. Ct values of each gene and treatment were used to determine fold induction of the gene according to the ΔΔCt method (Lehmann and Kreipe, 2001; Livak and Schmittgen, 2001), analysis of variance was performed and significant differences between means were determined by LSM using SAS ($p<0.05$). Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

Primer specificity verification

In order to verify the length of the quantitative PCR products, the amplicon size was verified by separation on a 2% agarose E-gel, staining with ethidium bromide and analysis under UV light (Invitrogen, Carlsbad, CA).

Results

Separation pattern of secreted proteins obtained by SDS-PAGE

The gel showed at least four different bands that appeared significantly darker in the supernatant from LPS-stimulated cells compared to control cells. The apparent molecular weights of these bands were approximately 200, 150, 100, and 70 kD. These bands were carefully excised and submitted for analysis by mass spectrometry. Further comparison of these bands 30 minutes, 3 hours and 24 hours after LPS stimulation did not reveal noticeable density differences between these time points, suggesting that after

the initial secretory event, no further accumulation with time had taken place (Figure 34).

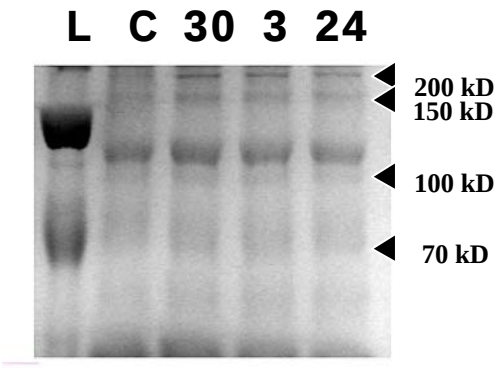


Figure 34: Supernatants from the LPS stimulated adherent cell population run on a polyacylamide gel. The samples were run on a 5% SDS polyacrylamide gels under reducing conditions. The Ladder (L) is in the first lane, the control supernatant (C) is in the second lane, and the 30 minute (30), 3 hour (3) and 24 hour (24) are in lanes 3, 4, and 5 respectively. Notice the almost immediate protein accumulation at 30 minutes of 200kD, 150kD, 100 kD, and 70 kD protein bands.

Protein sequence data

The protein electrophoresis indicated several proteins differentially expressed in the LPS treated samples compared to the control samples (Table 6). From the excised bands of the polyacrylamide gel, nine putative protein products were identified from the *in vitro* stimulation with LPS. Complement components C3, complement Factor B precursor, and complement Factor B subunit Bb were found to be at a higher levels during LPS stimulation. Other proteins such as trypsin and protease inhibitors as well as

Alpha-2-Heat Shock protein appeared to be differentially secreted during stimulation compared to control levels.

Table 6: The protein analysis results from mass spectrometry sequencing of the LPS stimulated adherent cell population. The molecular weight was determined using the published sequence and the molecular weight calculator found at the URL: http://bioinformatics.org/sms/prot_mw.html
(P=Pig, O=Orangutan, B=Bull, M=Mouse, H=Golden Hamster, S=Sheep)

Protein	**Molecular Weight**	**Approximate Size on Gel**	**PubMed ID**
Complement Component C3	187 (P)	200	gi|11869931
Inter alpha-trypsin inhibitor, heavy chain 4; PK-120 precursor	105 (M)	150	gi|9055252
Inter-alpha-trypsin inhibitor heavy chain H4 precursor (ITI heavy chain H4) (MAP Major Acute Phase Protein)	102 (P)	150	gi|3024051
Complement Factor B precursor C3/C5 Convertase (Properdin factor B)	85 (O)	100	gi|29690183
Complement Factor B subunit Bb	20 (P)	100	gi|543095
Inter-alpha-trypsin inhibitor heavy chain H3 precursor (ITI heavy chain H3)	99 (H)	100	gi|3024063
Alpha-2-HS-Glycoprotein	40 (B)	70	gi|27806751
Serine Proteinase Inhibitor - serine (or cysteine) proteinase inhibitor, clade A (alpha-1antiproteinase, antitrypsin), member 1	46 (B)	70	gi|27806941
Alpha-1-Antiproteinase precursor (Alpha-1-antitrypsin (Alpha-1-Proteinase inhibitor)	46 (S)	70	gi|112890

Quantitative PCR data

Using the protein data as a guide, we performed quantitative PCR on the cell fraction of the 3 hours stimulated samples. We evaluated the gene expression of the complement components, innate immune components, protease and trypsin inhibitors, heat shock proteins, as well as several apoptosis genes, and neuroendocrine components.

Primer verification

The agarose gel displays amplicons at the expected range for the primer pairs used (Figure 35). All bands were expected in the range of 70-200 base pairs in length, except A2HSP and C3, which were excluded from further analysis because primer pairs produced an amplicon approximately 450 base pairs in length, in addition to the expected 100 base pair amplicon. IL-12β primer verification is located in chapter V.

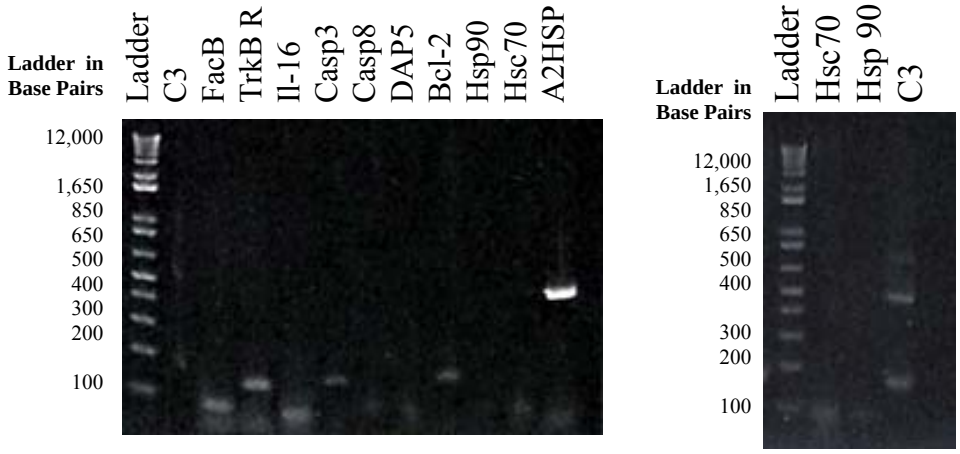
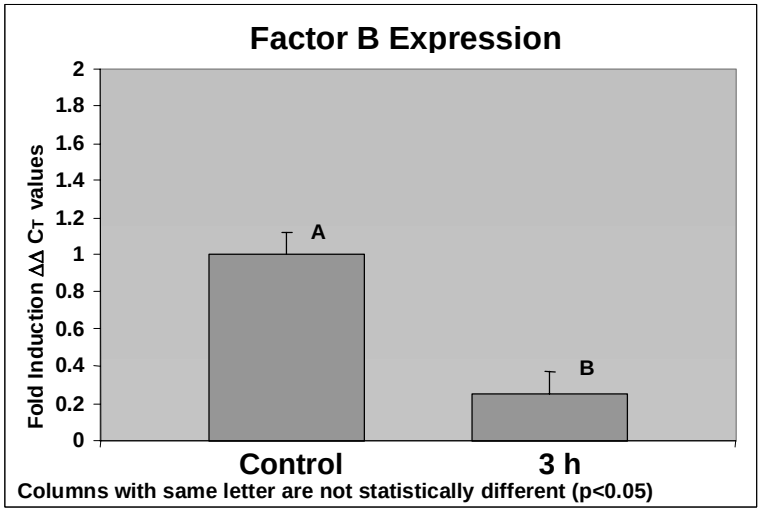


Figure 35: Amplicon size verification by 2% agarose gel electrophoresis of primers used for adherent cell population gene expression evaluation. Amplicons were expected between 80 and 200 base pairs in size. All amplicons are consistent with their expected specific base pair length, except A2HSP and C3 which were excluded from further analysis.

Complement components

Complement component C3 was excluded due to size exclusion on agarose gel electrophoresis. The gene expression levels of Factor B, were approximately 3.5 fold decreased at three hours post stimulation with LPS versus control level. (Figure 36)



36.

Figure 36: Quantitative PCR graphs showing the relative expression of Factor B of the 3 hour LPS stimulated adherent cell samples versus control samples. The gene expression levels of Factor B is markedly reduced in expression at 3 hours after stimulation. Each data point represents the average of 3 samples analyzed in duplicate ± SEM.

No significant differences were observed in the relative expression of any of the other genes included in the study.

Discussion

In the present study, the adherent cell population of the bursa of Fabricius, including the bursal secretory dendritic-like cells (BSDC), were enriched by adherence to plastic. The BSDC localization was confirmed by immunohistochemical staining with an anti-vimentin antibody, see chapter IV. The adherent cell population was stimulated with LPS from *Salmonella enterica* serovar Typhimurium and protein products secreted into the media were analyzed after thirty minutes, three hours, and twenty four hours of LPS exposure. The protein products were sequenced from the polyacrylamide gels, with the potential to identify co-migrating proteins as well as the differentially expressed proteins. To further characterize the secreted proteins, the samples needed to be confirmed via immunoassay or through evidence of mRNA production. Since specific antibodies were not available, the putative secreted proteins were used as a guide to choose genes to evaluate expression on a molecular level. Quantitative polymerase chain reaction (QPCR) was performed on purified RNA samples of the 3 hours sample group and compared to gene expression levels of the control (growth media only) sample. After 3 hours, the gene expression levels in the treated group were equal to or below those of the controls, including genes studied based on in vivo stimulation experiments from chapter V (results not shown). QPCR results display a snapshot of the gene expression levels at three hours post stimulation.

In this study we observed a new and exciting phenomenon, a burst of protein release by the non-lymphoid, adherent cell population of the bursa in response to LPS

stimulation. The most economic option of identification protein secretion was utilized, but further analysis using 2D electrophoresis is required to confirm specific protein production by these cells.

Surprisingly, there was no evidence of *de novo* synthesis of mRNA corresponding to proteins that are released initially following LPS stimulation. LPS alone does not seem to induce gene shifts, just a temporary activity of secretion without a detectable attempt to produce more of the released proteins. The initiation of protein production may require signals, such as IL-12 or Interferon γ, from surrounding cells that are not present in this artificial in vitro model (Puccetti et al., 2002). Previous studies have shown that a disruption in the cellular architecture of the bursa of Fabricius, such as making a single cells suspension indeed prevents the majority of immune interactions from occurring (Compton and Waldrip, 1998; Neiman et al., 1994). The B-cells are clearly dependent on signals or contact from the stromal cell population and *vice versa*. Our results seem to suggest that the adherent cells need signals from the B-cell population to induce protein production. A recently discovered member of the TNF family, BAFF, is an important signal for B-cell survival, both increasing the production of anti-apoptotic proteins via NF-κB mediated pathways and preventing genomic changes that induce apoptosis (Koskela et al., 2004; Mecklenbrauker et al., 2004; Schneider et al., 2004). The signal for protein release and *de novo* synthesis of the adherent cell population seem to be under differential control. Upon stimulation with LPS, pathogen-associated molecular patterns (PAMPs) may signal the release of the proteins, while the signal for synthesis of the protein comes from a different source.

The release of complement-related proteins following LPS stimulation is in accordance with current knowledge. LPS initiates the alternate pathway of the complement cascade, causing the release of Factor B from various cells including tissue macrophages as well as the cleavage of C3 to its functional parts and propagation of the cascade. The initial exposure of C3 to LPS activates the alternate pathway of the complement cascade by cleaving C3 into its active components, C3a and C3b. C3a is an inflammatory peptide that increases immune response, while C3b is an opsinin that continues the complement cascade by joining with Factor B to create a convertase that leads to the formation of the membrane attack complex (MAC). MAC then causes lysis of the invading organism.(Nauta et al., 2004) In this study, upon stimulation with LPS, an initial release of complement factors C3 and Factor B were detected by protein analysis as expected from the adherent cell population, which contains some tissue macrophages. The QPCR analysis shows Factor B expression to be significantly decreased compared to control levels by 3 fold. The decreased expression of Factor B makes sense under the experimental conditions, complement production is an immediate and short term event. If the levels of Factor B were to remain at an elevated level, the complement cascade would continue at a rapid pace causing unnecessary inflammation and immune response (Parham, 2000).

In summary, this study evaluated an enriched cell population from the bursa of Fabricius from which the B lymphocyte population was removed. Stimulation with LPS caused the potential initial release of complement component Factor B by the adherent cell population, followed by a decrease in gene expression 3 hours after stimulation.

Complement components were released shortly after stimulation and the gene expression was down regulated by 3 hours after initial stimulation, a pro-longed expression would cause unnecessary inflammation and tissue injury that would be detrimental to the system. In conjunction with complement components, the expression of pro-inflammatory cytokines did not indicate increased production either by LPS recognition by PAMPs on the APCs or apoptotic events influencing dendritic cell response. The LPS recognition and initial release of pro-inflammatory cytokines may occur, but the production of the proteins seems to be under different regulation. The apoptotic gene expression stayed constant at 3 hours post stimulation with LPS, and can be explained by the fact that the bursal B-cells that have a high rate of apoptosis are not components of this cell culture and the LPS signal does not induce apoptotic changes in the adherent population. The experimental model, the enriched cell population, is a valuable tool for the characterization of the adherent cells, but not their cellular interactions during immune stimulation with LPS. Like this one, future studies will be able to evaluate the protein release of the adherent, non-lymphoid cells from the bursa which has previously been impossible.

The confirmation of the protein release would require further studies, using a more profound proteomics approach using isoelectric focusing to separate the proteins in two dimensions, giving a purer protein product to analyze. Other options for further evaluation include identification of cross-reactive antibodies to chicken proteins to confirm protein release. Also, future experiments should include the recently discovered

B-cell survival and potential activator of stimulation, BAFF, in order to determine if it is released or produced by the adherent cell population.

CHAPTER VIII

DISCUSSION AND CONCLUSIONS

Our study of neuroendocrine-immune interactions in the chicken began by the localization of two molecules that were shared by both immune and endocrine tissues. First, as described in chapter II, **chromogranin A** (CgA), a classic neuroendocrine cell marker (Huttner et al., 1991a), appeared to be localized not only in the pituitary (Proudman et al., 2003), but also in the immune system of the chicken (Oubre et al., 2004). In the thymus of the chicken, the central organ of the cell-mediated immune system, immunohistochemical experiments displayed abundant staining of the diffuse neuroendocrine system with a new monoclonal antibody against CgA (Oubre et al., 2004). Somewhat surprisingly, attempts to localize CgA in the bursa of Fabricius, the central organ of the humoral immune system unique to birds, revealed very limited positive cell staining in healthy animals, restricted to very few cells and only visible with amplified enzymatic staining technology. Apparently, a readily detectable staining was only observed after severe tissue damage (B-cell decimation) by Infectious Bursal Disease Virus (IBDV) (results not shown). The second molecule that represented a potential immuno-neuroendocrine link in the resting avian immune system was **ovoinhibitor** (OI). Ovoinhibitor is a serine protease inhibiting protein that was originally purified from egg whites (Matsuda et al., 1983). In collaboration with the lab of Dr. Billy Hargis, we had localized this molecule for the first time in the context of the

immune system, *i.e.* in the bursa of Fabricius (Moore et al., 2004). The ovoinhibitor-positive cells in the bursa of Fabricius were mainly localized in the cortico-medullary border region of the bursal follicle, this is the same area as the **bursal secretory dendritic-like cells** (BSDC) (Moore et al., 2004). To further investigate the potential neuroendocrine nature of OI, immunohistochemical staining experiments using the anti-OI monoclonal antibody (Mab) were performed on the chicken pituitary and the brain. Chapter III discusses the co-localization of OI with the respective pituitary hormones. Dual staining analyses revealed co-localization of OI with luteinizing hormone, growth hormone and pro-opiomelanocortin, although the dual-stained cells were low in number compared to single stained (hormone-producing) cells. Molecular analysis confirmed the presence of an OI-related molecule in the pituitary by reverse transcription polymerase chain reaction and DNA sequencing procedures, although the amplicon obtained from pituitary mRNA was about 20% smaller than predicted from the previous reports of the ovoinhibitor sequence in the chicken oviduct (800 nucleotides instead of the expected 1000 nucleotides) (Scott et al., 1987). Also, the obtained cDNA sequence showed up- and downstream truncation of each of the represented exons, indicating alternative splicing. Previous reports of serpin genes have indicated alternative splicing as an efficient way to generate different inhibitors with varying target specificity (Kruger et al., 2002). This indicates the presence of an ovoinhibitor variant in the pituitary, but its function is still to be determined.

The observation that ovoinhibitor, a molecule we had localized in a neuroendocrine context, was localized in the bursa of Fabricius in the same region of the

bursa of Fabricius as the putative neuroendocrine cells, the BSDC (Moore et al., 2004), prompted us to further focus on the BSDC in a study of potential neuroendocrine-immune interactions in the maturation of the developing B-cells (Glick and Olah, 1987a; Moore et al., 2004). Previous studies had shown that the BSDC population can be labeled with an antibody against vimentin, an intermediate filament (Olah et al., 1992a; Olah et al., 1992b). Vimentin is classically known as a marker for the M-cells in the peyer's patches that transport antigens from the gut lumen to the underlying lymphoid cells (Ramirez and Gebert, 2003).

Immunohistochemical staining experiments of bursal tissue were performed, using antibodies specific for follicular dendritic cells and antigen-presenting cells. In addition, Laser Capture Microdissection (LCM) and flow cytometry were used in attempts to further characterize the BSDC (Chapter IV). LCM is an appropriate tool for collecting specific areas on tissue sections, such as the cortico-medullary border region of the bursa, but single cell collection, especially from immunostained tissue proved to be difficult to perform, especially for non-oval shaped cells, and quantities of RNA suitable for molecular analysis were not obtained. Therefore, flow cytometric fluorescence-activated cell sorting (FACS) was tested as an alternative option. Initial attempts at sorting the cells labeled with the antibody against vimentin, an intracellular antigen, indicated that the BSDC cell population represented less than 1% of the total bursal cell population. This is in agreement with previous electron microscopy analysis that show the BSDC population to be approximately 0.5% of the total bursal cell population (Olah and Glick, 1987). Unfortunately, the flow cytometry facility on Texas

A&M campus was not suitable to reliably sort a population of less than 1% of the total cell numbers, so a method for BSDC cell enrichment was required. Previous studies have demonstrated that 80% of the B-cell population dies within 6 hours of culture (Compton and Waldrip, 1998). Based on this information, single cell suspensions of the bursal tissue were made and either allowed to attach to plastic to yield an adherent cell population, or cultured overnight while shaking followed by density centrifugation on a Histopaque cushion to remove the B-cells and debris. For further flow cytometry analysis, the latter cell preparation was fixed and permeabilized for intracellular staining with a monoclonal anti-vimentin antibody (Dako Cytomation, Clone Vim 3B4, Carpinteria, CA). FACS analysis showed that density centrifugation after elimination of the B-cell population by apoptosis had resulted in a 10-fold increase in the vimentin-positive BSDC population. This provided crucial information with regard to the potential isolation of the BSDC. As a consequence of the fixation and permeabilization required for the intracellular staining of vimentin, the cells were obviously no longer usable for further culture and the RNA integrity was compromised preventing molecular analysis.

Several cell surface antibodies were used in attempts to sort the live BSDC cell population, but none were specific enough to obtain a pure BSDC population. Attempts were made using an anti-chicken IgG antibody (SouthernBiotech Birmingham, AL), an antibody supplied by Dr. Susan Jeurissen from the Central Veterinary Institute, Netherlands (CVI-ChNL-74.3) and an antibody given by Dr. Tom Scott from Clemson Univeristy (Mab 1A4). Thus far, the conclusion of these substantial purification efforts

inevitably seems to indicate that a new cell surface marker needs to be developed in order to allow for the efficient isolation of the BSDC cells from the rest of the bursal cell population by fluorescent or magnetic sorting. Scanning electron microscopy was also used to in an attempt to distinguish the BSDC among the various cell types in the adherent bursal cell fraction. Although various stellate cell types were observed, it appeared that the BSDC could not be reliably identified based on their morphological characteristics alone.

The next stage of this study was aimed at investigating the function of the bursa of Fabricius, more specifically its medullary compartment, in response to gut-derived or environmental antigens collected through cloacal reverse peristalsis (Ekino et al., 1980; Schaffner et al., 1974). It has been shown that environmentally collected antigens are very efficiently transported to the bursal lumen and internalized by the FAE (Ekino, 1993; Glick and Olah, 1993b; Naukkarinen and Sorvari, 1984). These antigens, in combination with maternal antibodies, appear to form immune complexes which are then presented by the BSDC to the surrounding B-cells, much like the role of follicular dendritic cells in the germinal centers of mammalian secondary immune organs (Glick, 1991; Yasuda et al., 2002). For this purpose, *Salmonella enterica* serovar Typhimurium was used as a model antigen, because it could be used for the inoculation with live bacteria *in vivo,* and LPS from the same bacteria could be used as a model antigen in the *in vitro* bursal adherent cell culture system described above. While the *in vivo* model provided a complete physiological system (Chapter V), the *in vitro* system would allow

us to focus exclusively on the role of stromal cells (including the BSDC), since all B-cells had been eliminated from the crude total bursal cell suspensions (Chapter VII).

The evaluation of the *in vivo* stimulation with live *Salmonella enterica* serovar Typhimurium was further explored in chapter V. QPCR analysis displayed a rapid and high gene expression of IL-12 during Salmonella challenge of the intact *in vivo* system, that was absent from the LPS stimulation of the adherent cell population. This is the first reporting of IL-12 up-regulation *in vivo* in the chicken. The B-cells are the likely source of the IL-12 production in the total bursal population because the expression of IL-12 in the B-cell devoid *in vitro* experiments did not display a change in IL-12 production after stimulation with LPS. This is in line with current findings of IL-12 expression of LPS-stimulated DT40 cells, avian B-cell line (Degen et al., 2004). The other interesting outcome of this study was the shift of gene expression of apoptosis related genes to increased apoptosis. The apoptosis antagonist, Bcl-2, was dramatically decreased by 24 hours post challenge while the initiator, caspase 8 and executor, caspase 3, of apoptosis were up-regulated. This shows the apparent trend towards apoptosis following challenge with an intracellular pathogen up to 24 hours post-challenge. In this experiment, the *in vivo* total bursal sample showed a modest decrease in protease inhibitor gene expression, while the *in vitro* enriched cell sample displayed drastic decreases in protease inhibitor gene expression. As a result of LPS stimulation, the adherent cell population decreases the expression of serine protease inhibitors, which in turn may increase the enzymatic activity of the proteases themselves, although current knowledge is lacking on the actual enzymatic cognate of these protease inhibitors.

Further investigation is required to understand the relevance of this decrease in mRNA expression due to LPS stimulation. In the case of ovoinhibitor gene expression, the levels decrease to a point where the gene expression was almost non-existent compared to control levels, and in addition, any residual ovoinhibitor mRNA had been degraded. The combination of the decrease in OI and the localization of OI positive cells in the bursa, indicate a potential role for OI and the BSDC in response to LPS stimulation. It is tempting to speculate that the cognate enzyme of OI is part of the secretory granule contents of the BSDC.

The adherent cell enrichment was used to evaluate protein production and expression following *Salmonella* LPS stimulation (Chapter VII). A potential initial release of proteins related to innate immunity, including complement component, heat shock proteins and protease inhibitor were detected by protein analysis of cell supernatants collected from the *in vitro* enriched adherent cell population selected by adherence to plastic. Quantitative polymerase chain reaction (QPCR) was used to identify if a up-regulation of mRNA expression occurred along with protein release. The protein production of most of the genes evaluated were not significantly different as compared to the control expression.

In summary, the development of a novel method for the enrichment of the non-lymphoid cell population in the bursa of Fabricius is a major contribution of this work. Furture studies will be able to utilize these methods for continued characterization of the BSDC population. It is well known that the stromal cells play a pivotal role in the maturation and differentiation of B lymphocytes, so further investigation into cells in the

stromal population will give insights into humoral immune function. The chicken has again been proven to be useful for the study of the development of the humoral immune system, and many of the findings can be carried over into other species, including humans, just as the original identification of the division of the humoral and specific immune system was applied to other species (Glick, 1987). The information gained using the newly developed method will contribute to the knowledge and understanding of the humoral immune system. Other contributions include the identification of chromogranin A in the thymus providing another link between the immune system and the neuroendocrine systems. The localization of ovoinhibitor in the pituitary and its decrease in gene expression levels in the adherent cell population from the bursa of Fabricius following LPS stimulation was also identified during this study.

For the first time, IL-12 mRNA production has been shown *in vivo* in the chicken, but not in the adherent enriched, B-cell depleted, *in vitro* cell population. The utilization of the newly developed artificial in vitro systems will help to further dissect the roles that individual cell types play in the ongoing neuroendocrine-immune response interactions.

The biggest limitation to the study of the BSDC population is the isolation of the cell population. Obtaining a pure BSDC cell population would allow the proper classification of these cells, whether they are true or modified dendritic cells and help elucidate their true function in the bursa and the immune system as a whole. The study of the Bursal Secretory Dendritic-like Cells is expected to give us an insight into a new level of regulation that has not been previously recognized. The elucidation of avian

neuroendocrine-immune response reactions will increase our overall knowledge in these fields. The results can potentially aid in immunotherapeutic endeavors and design of more effective vaccine strategies.

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Selected publications

- Ovoinhibitor in the chicken bursa of Fabricius: identification, isolation, and localization.
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